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14. ABSTRACT Results continued to accumulate that are consistent with the hypothesis that disfacilitation of wake-promoting systems by the hypocretin (Hcrt) receptor antagonist almorexant (ALM) results in less functional impairment than the inhibition of neural activity produced by the benzodiazepine receptor agonist zolpidem (ZOL). In Year 4, the previously-reported results on spatial reference memory (Task 2a) and spatial working memory (Task 2b) were complemented by Psychomotor Vigilance Test studies (Task 2c). The wake-active histaminergic and Hcrt neurons, but neither the cholinergic basal forebrain (BF) neurons nor the serotonergic dorsal raphe neurons, could be activated after sleep deprivation in the presence of ALM; however, none of these four cell types could be activated in the presence of ZOL (Task 3a). Lesions of the locus coeruleus (LC), a wakefulness-promoting area, abolished the ALM-induced decrease in NREM sleep latency without affecting the ZOL-induced decrease (Task 3b). High sleep pressure, rather than the actions of ALM or ZOL per se, is critical for activation of sleep-active cortical neurons (Task 4a). ALM promoted adenosine release in the BF (Task 4b) and cortex (Task 4c), particularly during waking. Hcrt neurons expressing channelrhodopsin-2 can be excited by blue light pulses in vitro and preliminary in vivo experiments indicate that optogenetic activation of Hcrt cells can cause changes in sleep architecture (Tasks 6a).					
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PROGRESS REPORT

“Effect of a Hypocretin/Orexin Antagonist on Neurocognitive Performance”

USAMRAA Grant W81XWH-09-2-0081

DR080789P1

Year 4: 8/1/12 to 7/31/13

Thomas S. Kilduff, Ph.D., Principal Investigator

INTRODUCTION

Almorexant (ALM) is a hypocretin/orexin (Hcrt) receptor antagonist with a novel mechanism of action that has shown promise as an effective hypnotic. Preclinical data demonstrate that animals treated with ALM are easily aroused from sleep and are free of ataxia and other behavioral impairments. If this observation is confirmed in humans, it would have enormous implications for the management of disturbed sleep in both military and civilian populations. The overall hypothesis that underlies this research is that ALM produces less functional impairment than the benzodiazepine receptor agonist zolpidem (ZOL) because ZOL causes a general inhibition of neural activity whereas ALM specifically disfacilitates wake-promoting systems. Whereas the human study component will establish whether ALM is superior to ZOL in neurocognitive tests, the animal studies will compare the neural circuitry that underlies the activity of these compounds, their effects on sleep and performance, and the effects of these compounds on biomarkers associated with normal sleep.

BODY

Task 2. *Test the hypothesis that rodents receiving ZOL will show greater neurocognitive impairment than those receiving ALM or PBO.*

- 2a. Assessment of Almorexant effects on spatial reference memory in rats: Data collection and analysis completed; manuscript preparation ongoing.
- 2b. Assessment of Almorexant effects on spatial working memory in rats: Data collection and analysis completed; manuscript preparation ongoing.
- 2c. Assessment of Almorexant effects on psychomotor vigilance in rats: Data collection completed; analysis ongoing.
- 2d. Synthesis of ALM (months 1-4). COMPLETED

Progress – Task 2a: All data collection and analysis for Task 2a is complete. All water maze (WM) data for the effects of ALM on spatial reference memory are reported here. We have assessed the effects of ALM on spatial reference memory both following undisturbed and sleep deprivation (SD) conditions.

Methods: All rats were given a minimum of 3 wks for recovery from surgery and each rat was recorded for a 24 h period to determine basal sleep/wake patterns. Completion of this Task was delayed as it was necessary to use 100 rats (including those used for pilot studies) instead of the 72 originally proposed. Assessment of the effects of ALM on spatial reference memory occurred on 2 consecutive days. On day 1, the acquisition of the task occurred in one session consisting of 8-12 consecutive WM trials with a 60 second (s) inter-trial interval. On the following day, rats were either left undisturbed and dosed with ALM, ZOL or vehicle (VEH) 6 h into their active period (ZT18) or kept awake for the first 6 h of the dark and then dosed. Subsequent to dosing, rats were left undisturbed for 90 min and then a retention probe trial was

performed. For this test, the platform was removed from the WM and the rats were allowed to swim and search for the platform for 30 s. Parameters measured during the retention probe trial included the time and distance traveled in the quadrant of the WM where the platform had been on the acquisition day, as well as the latency and the number of entries into the target quadrant. EEG and EMG recordings were analyzed from the beginning of lights out (ZT12) until initiation of the WM test (7.5 h later). For more details on our experimental procedures, please see the full protocols in our original proposal.

Results: As reported previously, both ALM (100 mg/kg i.p and p.o.) and ZOL (30 mg/kg i.p. and 100 mg/kg p.o.) had significant sleep-promoting effects. Waking (W) was decreased and non-rapid eye movement sleep (NR) increased by ALM and ZOL compared to vehicle. Confirmation of these effects are presented in Figure 1. Following undisturbed conditions, rats administered ALM and ZOL slept equivalent amounts for the last 60 min prior to WM testing (Figure 1A). Interestingly, following 6 h SD, rats administered ALM slept more than rats administered ZOL during the 60 min prior to testing.

During the WM probe trial following undisturbed conditions, rats administered ZOL showed impairments in all parameters measured compared to rats administered VEH or ALM whereas ALM was indistinguishable from VEH for all measures (Figure 2). Following ZOL, rats swam significantly less (Fig. 2A), took longer to reach the target zone (Fig. 2C), spent less time in the target zone (Fig. 2E), and entered the target zone less frequently (Fig. 2G) compared to rats administered VEH or ALM.

During the WM probe trial following the 6 h sleep deprivation, ALM was again indistinguishable from VEH for all measures. Following ZOL, rats swam significantly less (Fig. 2B) and took longer to reach the target zone (Fig. 2D) compared to rats administered VEH or ALM. However, time spent in the target zone (Fig. 2F) and the frequency of entering the target zone (Fig. 2H) was not different from rats administered VEH or ALM.

These results are consistent with the hypothesis that, although both ALM and ZOL are effective hypnotic medications, rats would show less functional impairment following ALM than following ZOL treatment.

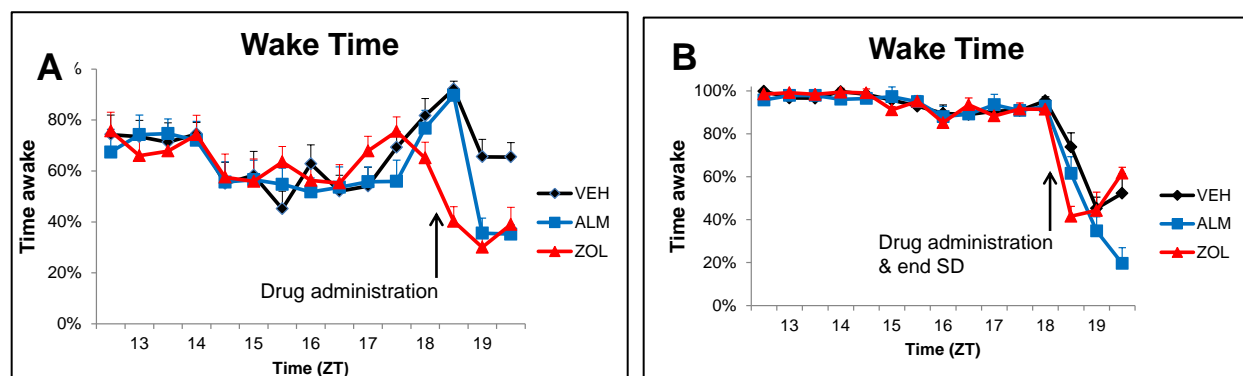


Figure 1. Percent time awake for 6 h prior and 90 min following drug administration (p.o.) following undisturbed conditions (A) and following 6 h SD (B). **A.** Rats administered ZOL went to sleep more rapidly than rats administered ALM but for the last 60 min prior to WM testing ALM and ZOL rats slept equivalent amounts. **B.** Following 6 h SD, rats administered ALM slept more for the 60 min prior to WM testing compared to ZOL-treated rats.

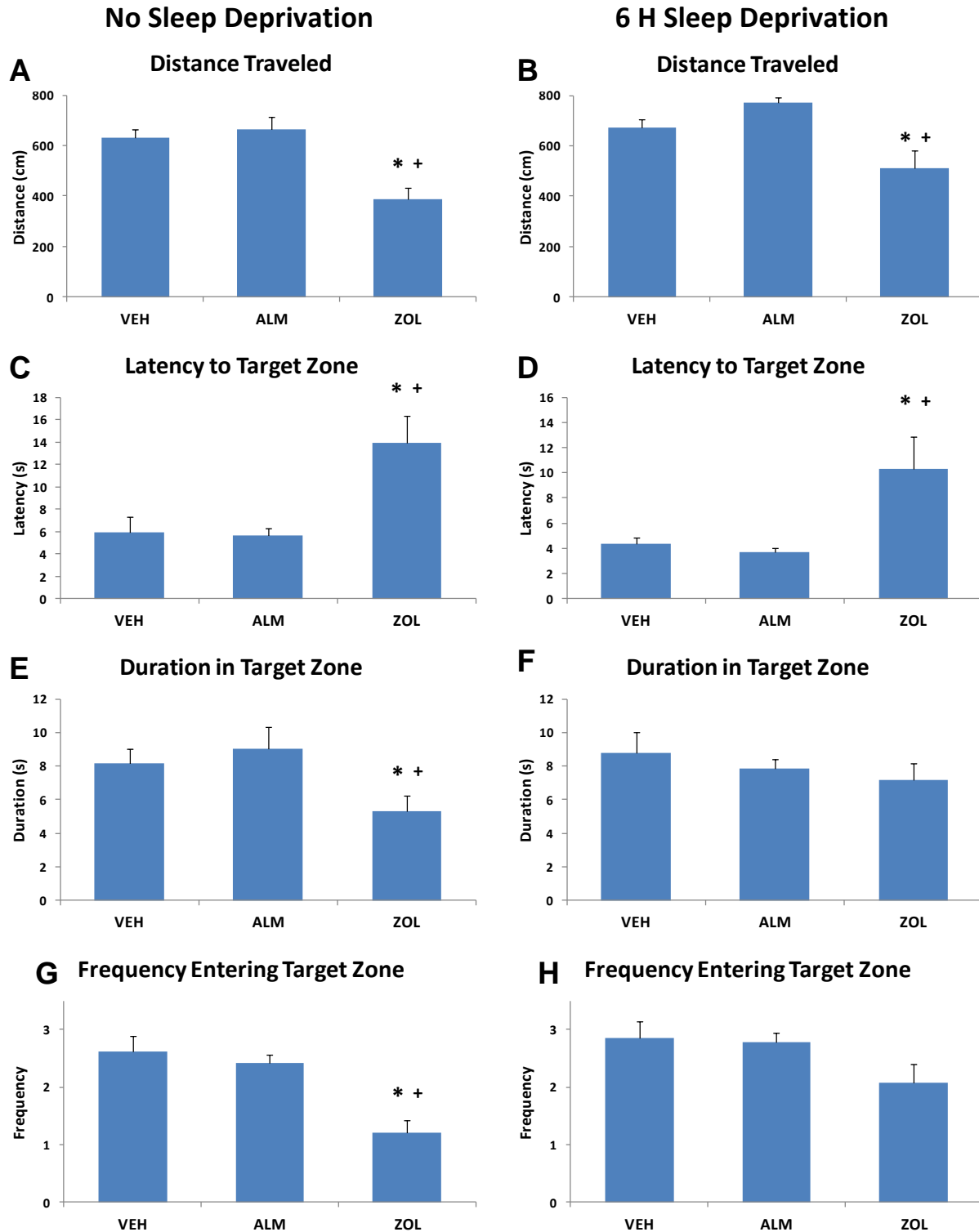


Figure 2. Measures of spatial reference memory during the WM probe trial. For the non-sleep deprived condition (left column), rats were left undisturbed for the first 6 h after lights off. Rats were then dosed and tested 90 min later. For the sleep deprived condition (right column), rats were kept awake for the first 6 h after lights off and then dosed. Following dosing, rats were left undisturbed and tested 90 min later. **A, B.** Distance traveled during the 30 s probe trial. Rats administered ZOL swam significantly less than rats administered vehicle or ALM following both undisturbed and sleep deprived conditions. **C, D.** Latency to

entry into the target zone. Rats administered ZOL took significantly longer time to enter the target zone compared to rats administered vehicle or ALM following both undisturbed and sleep deprived conditions. **E, F.** Time spent in the target zone. Rats administered ZOL spent significantly less time in the target zone compared to rats administered vehicle or ALM only following undisturbed conditions. **G, H.** Frequency of entry into the target zone. Rats administered ZOL entered the target zone significantly fewer times compared to rats administered vehicle or ALM only following undisturbed conditions.
* = significantly different from vehicle; + = significantly different from ALM.

Progress – Task 2b: Similar to the assessment of the effects of ALM on spatial reference memory in Task 2a, we have proposed to assess the effects of ALM on spatial working memory following both undisturbed and sleep deprived conditions in Task 2b. All data collection and analysis for Task 2b is complete. All WM data for the effects of ALM on spatial reference memory are reported here.

Methods: The general protocol for assessing spatial working memory is as follows. Approximately one week before spatial working memory testing, rats are trained in a standard WM protocol as described in Task 2a. During this training, the location of the hidden platform remains constant across all trials. A baseline sleep recording occurs following training but prior to testing, in order to assess the basal sleep-wake patterns of each rat. On the experimental day, rats are either left undisturbed prior to administration of drug at ZT18 or sleep deprived for 6 h prior to drug administration. Rats are then left undisturbed until testing 60 min later.

The spatial working memory task consists of 6 pairs of trials. In the first trial, a cued platform marked with a flag is placed in one of 6 positions in the tank. Rats are released facing the wall in one of the 3 quadrants that does not contain the platform and are allowed 120 s to locate the cued platform before the researcher guides the rat to the platform. After 15 s on the platform, rats are removed from the WM and placed in a holding cage. The flag is then removed but the platform remains in the same location as in the first trial. Following a delay of 1, 5, or 10 min in the holding cage, the rat is placed back in the tank in one of the two quadrants that did not contain the platform and was not the starting quadrant from the first trial. Once the rat finds the platform, it is removed after approximately 5 s on the platform and placed back in a holding cage for 10 min before a new pair of trials with a novel platform location is given. The order of delays is counterbalanced so that each rat is tested twice at 1, 5, or 10 min delays between the cued and hidden platforms. Test measures are velocity, time and distance traveled to locate the platform during all tests.

Results: ZOL-treated rats showed impairments in velocity and in the time to locate the platform after both the undisturbed and 6 h SD conditions (Figure 3). In contrast, ALM-treated rats were indistinguishable to VEH-treated rats for both velocity and in the time to locate the platform after both the undisturbed and 6 h SD conditions. Importantly, ZOL rats only found the platform 33-50% of the time while ALM and VEH rats found the platform 79-100% of the time VEH (Figure 4). These results demonstrate that ALM impairs the performance of rats less than ZOL does in this spatial reference memory task.

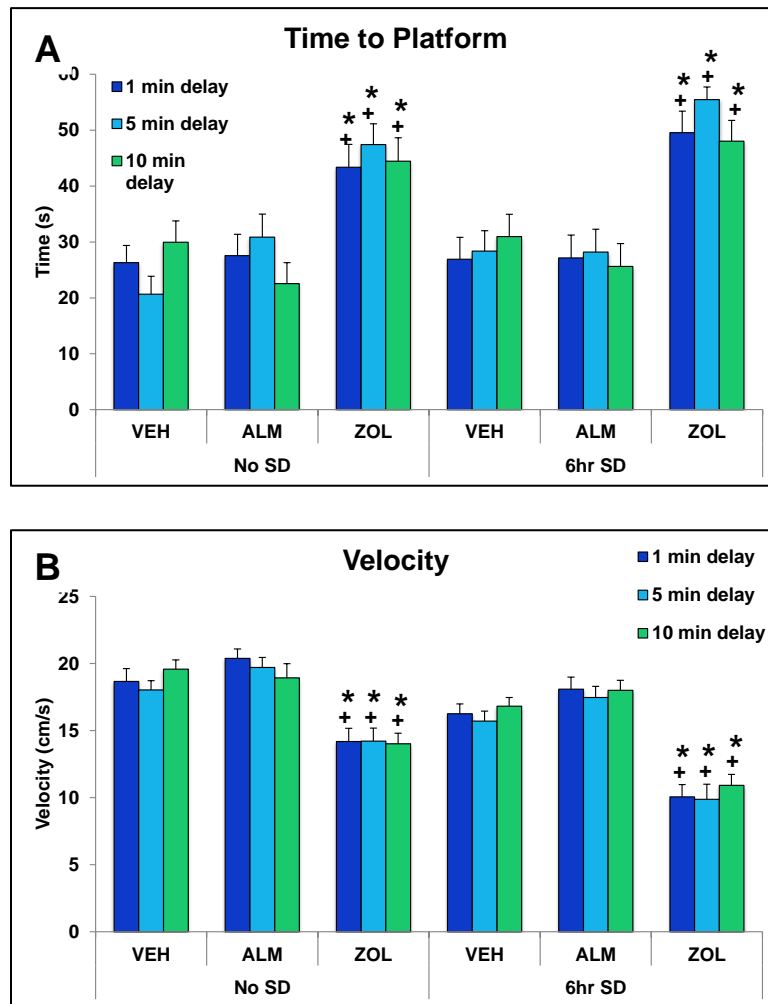


Figure 3. Measures of spatial working memory during the WM task following undisturbed (left) and sleep deprivation (right) conditions. **A.** Time taken to find platform during the 60 s test trial following undisturbed and sleep deprivation conditions. Rats administered ZOL took significantly longer to find the platform than rats administered vehicle or ALM. **B.** Velocity of the rat during the 60 s test trial. Rats administered ZOL swam significantly slower than rats administered vehicle or ALM following undisturbed and sleep deprivation conditions.

* = significantly different from vehicle; + = significantly different from ALM.

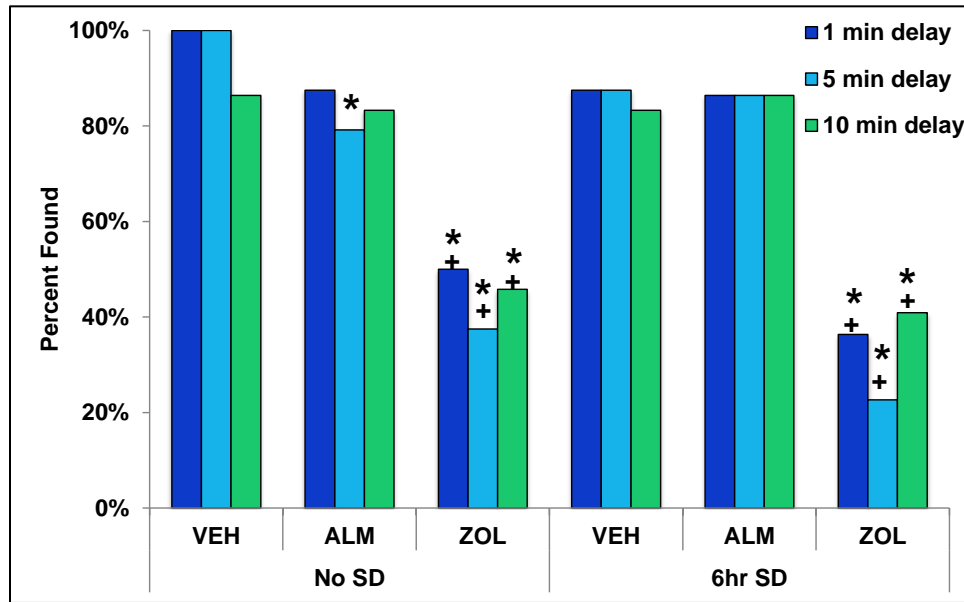


Figure 4. Percentage of trials during which rats found the platform during the WM test trial measuring spatial working memory. Rats administered ZOL found the platform significantly less than rats administered vehicle or ALM following undisturbed (left) and sleep deprivation (right) conditions. * = significantly different from vehicle; + = significantly different from ALM.

Progress – Task 2c: The equipment for the rodent psychomotor vigilance (rPVT) test arrived in November of last year. However, the equipment arrived with an incorrect reward system so we did not have a functional system until January of this year. The experiments commenced starting in February and were completed in July. Data analysis is ongoing.

Methods: The general protocol for the rPVT is as follows. Rats were motivated to perform the operant rPVT task for water reinforcement by having water unavailable to them for 23 h prior to all operant training and testing. Rats were gradually acclimated to the water restriction schedule over several days by reducing the amount of time each day that water was available in the home cage. rPVT training took 3 mo to complete. Following this 3 mo training period, rats that did not meet criteria (> 100 correct responses per test session) were removed from the study. rPVT testing consisted of a stimulus light on for a duration of 0.5 s followed by a 3 s response period. The intertrial interval varied between 3-7 s. Errors resulted in a 10 s “time out” period during which the dim house lights were turned off. Test measures were response latencies, correct responses, omissions, premature responses, lapses and the number of trials.

Results: Seventeen rats were implanted with telemetry devices for EEG recordings. Of these, 4 rats did not meet criteria following 3 mo of training and were removed from the study. We anticipated that up to a third of the rats might not meet criteria so these results were expected. In addition, 2 rats had transmitter malfunctions prior to completion of the study and could not be included in our results. Therefore, 11 rats completed the rPVT study.

When the testing was about to begin, rats were acclimated to the dosing procedure by administration of 1 ml of VEH (p.o.). However, when we examined the performance following this dose of VEH we found a significant decline in all rPVT measure. Therefore, we reformulated the VEH solution using a base of physiological saline rather than sterile water. This reformulation was effective at keeping the rats’ performance in the rPVT above minimum criteria following dosing with VEH.

When the experiments were initiated, it became clear very early on that there were deficits in performance following ZOL at 100 mg/kg. Some rats had very few responses to the stimulus following ZOL. In addition, ALM-treated rats showed a noticeable deficit compared to VEH. Therefore, we added two additional conditions: ALM and ZOL at 30 mg/kg (p.o.). These additional concentrations of ALM and ZOL have been shown to be sleep-promoting, but to a lesser extent than 100 mg/kg doses.

Although full analysis of the data has only just begun, very interesting effects are clearly present. Generally, ZOL-treated rats at both 30 and 100 mg/kg doses have increased response latencies, omissions and lapses and decreased correct responses, numbers or trials and premature responses compared to VEH following both undisturbed and SD conditions. The results following ALM administration are more complicated. While some measures do not appear different from VEH (such as response latencies), others show a decrease in performance with increasing dose concentration. However, these results are very preliminary and detailed analyses with statistics are ongoing.

Task 3. *Test the hypothesis that the Hcrt antagonist ALM induces sleep by selectively disfacilitating the activity of the histaminergic, serotonergic, noradrenergic and cholinergic wake-promoting systems whereas the B₂RA ZOL causes a generalized inhibition of the brain.*

3a. Double-label immunohistochemistry with Fos and phenotypic markers: Data collection nearly completed; analysis ongoing.

3b. Assessment of hypnotic efficacy in saporin-lesioned rats: Data collection to be completed by 8/31/13; analysis ongoing.

~~3c. Assessment of hypnotic efficacy in transgenic mice.~~

Progress -Task 3a: We proposed to determine whether ALM and/or ZOL interfered with activation of several major wake-promoting neuronal populations during a period of forced waking, using Fos immunoreactivity as a marker of functional activity of neurons. Data collection for Task 3a is nearly complete; histological analyses are ongoing. Completion of Task 3a was delayed because it was necessary to utilize 76 rats (including those used for pilot studies) instead of the 40 originally projected.

Methods: Rats were given 1 mL (p.o.) ALM (100mg/kg, n=16), ZOL (100mg/kg, n=16), or VEH (n=14) at their mid-active phase (ZT18). Half of the animals were left undisturbed for 1.5h after dosing, while the other half of the rats were sleep deprived (SD) by gentle handling for 1.5h. Animals were then deeply anesthetized and perfused, and the brains were removed and sectioned on a freezing microtome. Double label immunohistochemistry for Fos and markers for wake-active neurons (histamine (HA), hypocretin (Hcrt), serotonin (5-HT), and acetylcholine (ACh)) was performed using coronal sections of tissue from the appropriate brain region.

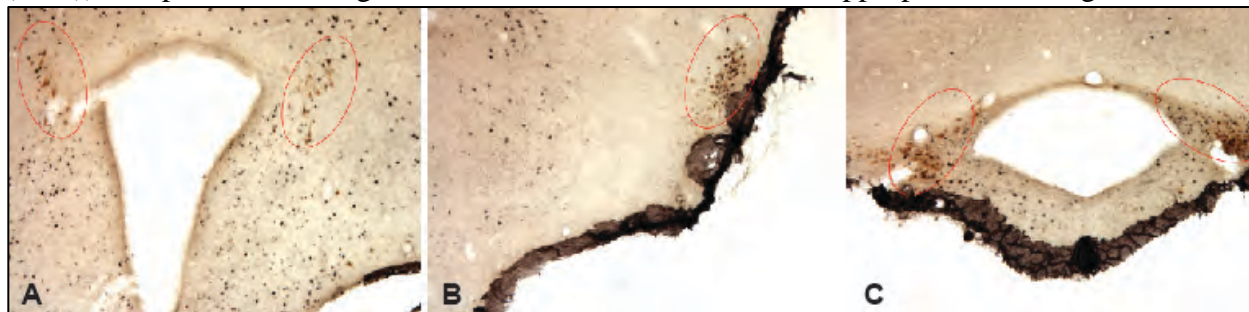
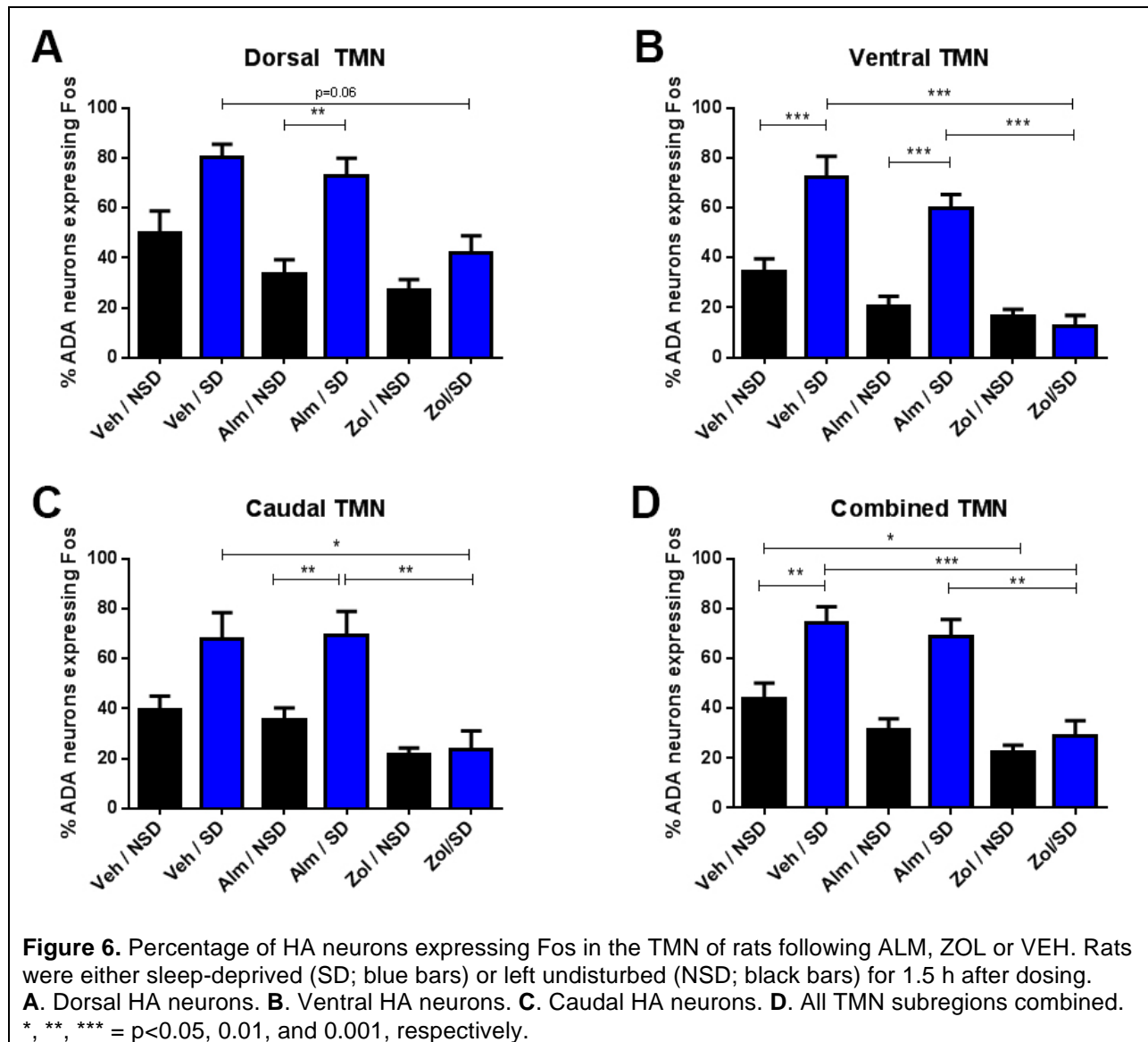


Figure 5. Fos-positive nuclei (blue-black stain) within ADA-positive cells (brown) indicate recent neuronal activation in three subregions of HA neurons in the tubermammillary nuclei. **A.** Dorsal HA neurons. **B.** Ventral HA neurons. **C.** Caudal HA neurons. The region of interest is circled in red.



Results: Double labeling experiments for Fos and adenosine deaminase (ADA), a marker for HA neurons, were scored for the three subpopulations of HA neurons: dorsal, ventral, and caudal (Figure 5). In all three regions, both VEH- and ALM-treated SD animals showed increased Fos expression in HA neurons compared to undisturbed animals (Figure 6), while ZOL-treated animals showed no increase in Fos expression following SD. In most cases, both VEH and ALM SD groups were significantly different from ZOL SD. These results indicate that activation of HA neurons is unimpaired by ALM whereas ZOL blocks SD-induced activation.

We conducted additional immunostaining and analysis of the Hcrt double-labeling experiments reported last year. This analysis confirms the preliminary finding (reported last year) that Hcrt neurons exhibit elevated Fos levels after SD in ALM-, but not ZOL-treated animals (Figure 7). These results indicate that ALM does not impair SD-induced activation of Hcrt neurons.

To investigate whether wake-active neurons in the basal forebrain are affected by ALM and ZOL, neurons that express choline acetyltransferase (ChAT), a marker for ACh, were scored for Fos co-expression. No significant differences were found between VEH-, ALM-, or ZOL-

treated animals in undisturbed or SD conditions, although a trend was found suggesting that SD animals treated with ALM may exhibit more Fos co-expression than do ZOL-treated animals after SD.

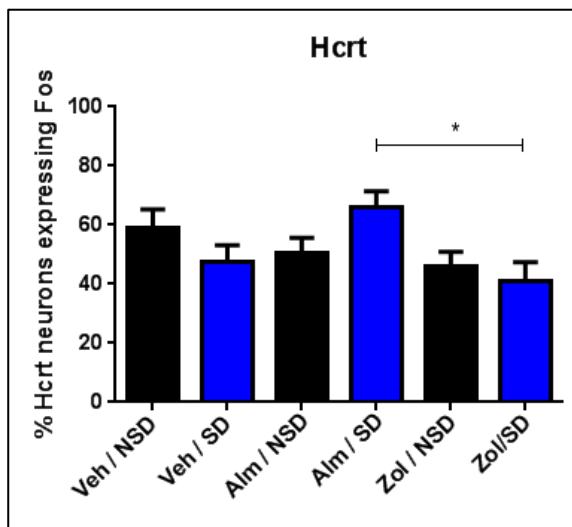


Figure 7. Effect of drug treatment on Fos expression in Hcrt neurons. following ALM, ZOL or VEH. Rats were either kept sleep-deprived (blue bars) or left undisturbed (black bars) for 1.5 h after dosing. *, $p < 0.05$.

Three subpopulations of 5-HT-expressing neurons in the dorsal raphe were scored for Fos double labeling. No significant changes in Fos expression were observed between VEH-, ALM-, or ZOL-treated animals under SD or undisturbed conditions, indicating that neither ALM or ZOL affect the activity of 5-HT neurons in the dorsal raphe.

Progress - Task 3b: We proposed to determine whether lesion of the subcortical wake-promoting neural populations would adversely affect the ability of ALM or ZOL to promote sleep. These Tasks 3b1, 3b2 and 3b3 required use of 32, 35 and 40 rats (including those used for pilot studies) instead of the 24, 24, and 26 rats originally planned. Consequently, completion of Task 3b has been delayed and incurred greater expense than originally projected. Data collection for two of the three lesion studies has now been completed, and we expect to complete data collection on the third by 8/15/13. Analysis of all three studies is now ongoing.

Methods: This year, we completed the second lesion study, examining the efficacy of ALM and ZOL in rats with or without bilateral lesions of the wake-promoting noradrenergic locus coeruleus (LC) using anti-DBH-saporin (DBH-SAP). 15 male Sprague-Dawley rats were instrumented for telemetric recording of EEG, EMG, locomotor activity (LMA) and core body temperature (CBT), and received an infusion of the neurotoxin DBH-SAP ($n=8$) or sterile saline ($n=7$) into the 3rd ventricle using a Hamilton syringe with a 26-gauge stainless steel needle coupled to a digital microinjection pump (World Precision Instruments, Sarasota FL). We verified that this technique reliably lesioned the LC in pilot experiments conducted last year. Rats were given 3 wk recovery to ensure that neuronal degeneration was complete. Prior to dosing, rats were recorded for a 24 h undisturbed baseline. Rats were given p.o. ALM 30, 100 and 300 mg/kg, ZOL 10, 30 and 100 mg/kg, and VEH at lights-off (ZT12) in balanced order with at least 3 d between treatments. EEG, EMG, LMA and CBT were recorded continuously throughout experimentation.

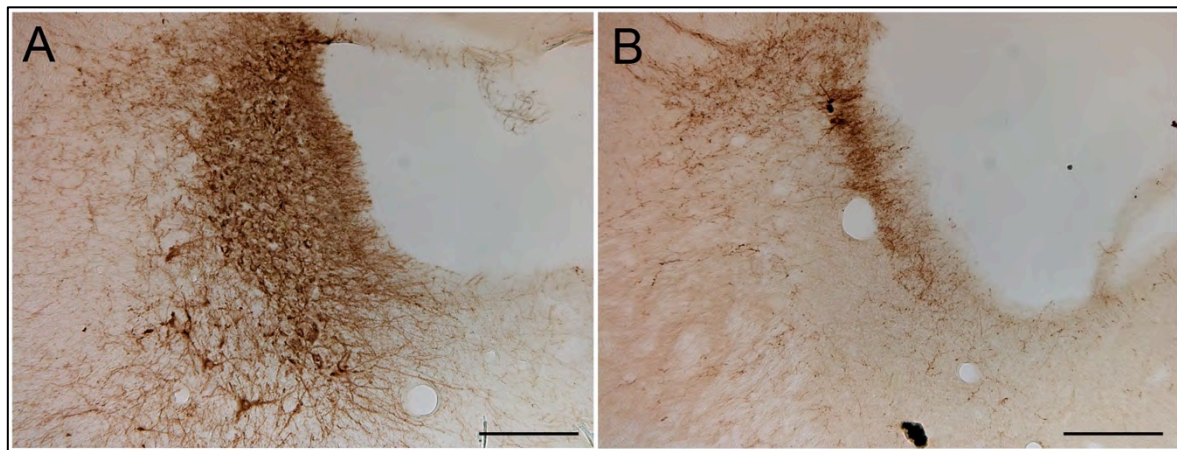


Figure 8. DBH-immunoreactivity in the locus coeruleus (LC) of rats. **A.** The LC is intact following injection of the neurotoxin 192-IgG saporin, which targets cholinergic neurons; DBH-immunopositive cells are numerous and densely packed, making it difficult to accurately count them. **B.** Only scattered DBH-positive neurons remain following injection of DBH-saporin; in this representative image, two cells are visible. Scale bar = 200 μ m.

Results: The LC was intact in saline-injected rats, appearing as a dense cluster of DBH-positive neurons (Figure 8A). In LC-lesioned (LCx) rats, the LC was entirely ablated (Figure 8B), with only scattered noradrenergic neurons remaining (15.75 ± 4.19 DBH-positive neurons per animal).

LCx attenuated ALM-induced decreases in NREM sleep latency, but did not alter ZOL-induced reductions in NREM latency compared to Sham rats ($F_{6,78} = 2.55$, $p = 0.026$; Figure 9A). ALM shortened REM sleep latency in both LCx and Sham rats ($F_{6,78} = 12.58$, $p < 0.0001$; Figure 9B). While there was not a significant effect of lesion condition on REM sleep latency, LCx rats exhibited a smaller magnitude change from vehicle compared to Shams, whereas ZOL increased REM latency to a similar extent in both LCx and Sham rats. These data indicate that lesioning the LC attenuated the sleep-inducing effect of ALM, but not ZOL.

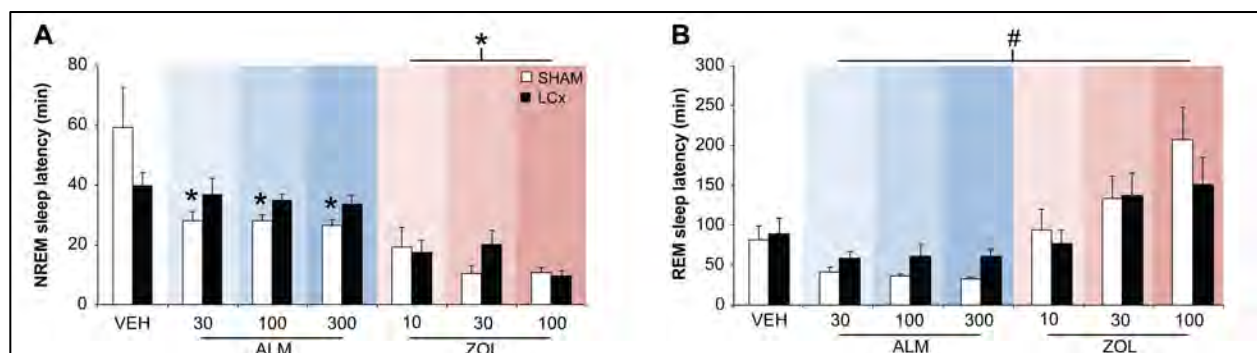


Figure 9. Sleep latencies in LCx (black) and Sham-operated (white) rats following administration of VEH, ALM (blue bkgd) and ZOL (red bkgd) at lights-out (ZT12). All doses in mg/kg. **A.** Latency to NREM sleep. **B.** Latency to REM sleep. *, significant drug x lesion interaction; #, significant main effect of drug only. All effects significant at $p < 0.05$.

LCx increased total NREM sleep duration independently of drug condition ($F_{1,13} = 5.72$, $p = 0.033$), but did not influence the effects of either ALM or ZOL, which increased NREM sleep compared to VEH at all doses ($F_{6,78} = 18.82$, $p < 0.0001$; Figure 10A). By contrast, LCx attenuated ALM-induced increases, but not ZOL-induced decreases, in REM sleep compared to Shams ($F_{6,78} = 4.44$, $p < 0.001$; Figure 10B). Similarly, LCx attenuated ALM-induced increases in REM bout number ($F_{6,78} = 4.52$, $p < 0.001$). These data demonstrate that LC lesions attenuate ALM-induced increases in REM sleep duration and bout number, but not ZOL-induced REM suppression.

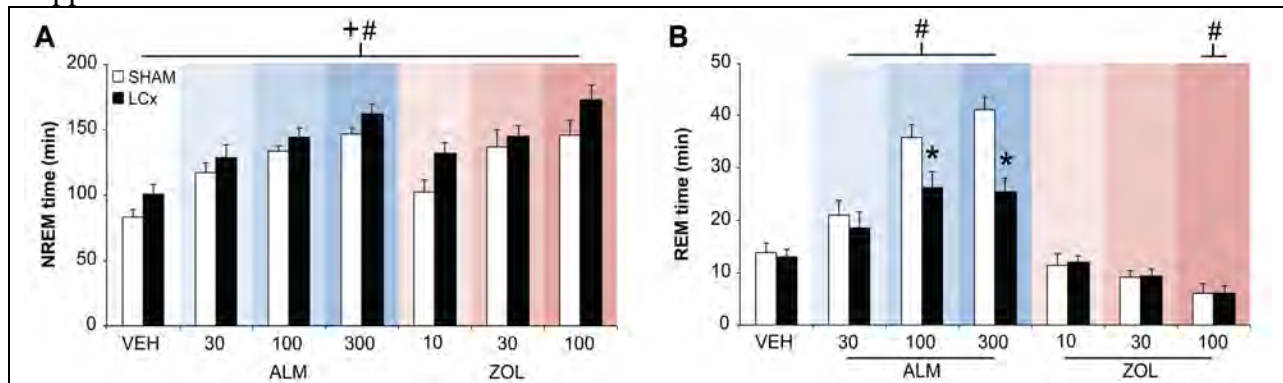


Figure 10. Cumulative sleep time in LCx (black) and Sham-operated (white) rats for 6 h following administration of VEH, ALM (blue bkgd) and ZOL (red bkgd) at lights-out (ZT12). All doses in mg/kg. **A.** Cumulative NREM sleep. **B.** Cumulative REM sleep. *, significant drug x lesion interaction; #, significant effect of drug only; +, significant effect of lesion only. All effects significant at $p < 0.05$.

In pilot studies, we verified our microinjection technique and the dosage for lesioning the wake-promoting tuberomammillary nuclei using Hcrt2-conjugated saporin (HCRT-SAP). HCRT-SAP was bilaterally injected directly into the posterior hypothalamus using a Hamilton syringe with a 30-gauge stainless steel needle coupled to a digital microinjection pump (World Precision Instruments, Sarasota FL). Infusions of 57-85 ng HCRT-SAP effectively lesioned the TMN, whereas infusions of unconjugated SAP or sterile saline left the TMN intact.

Progress - Task 3c: Despite considerable investment of efforts from 2011 to the present time, we have been unable to obtain the transgenic mice necessary to conduct this Task. Since the start of the project, we have identified three sources for the *Pet1-LMX1b* mice, two sources for the HDC-KO mice, and one source for DBH-KO mice. However, due to concerns over pathogen contamination, the IACUC at SRI International has refused to grant permission to allow us to import transgenic mice from three of those sources. Two sources encountered breeding problems breeding the mice (one lost his colony entirely) and one source was unresponsive to our requests, despite having written a letter of support for the original proposal. As indicated in our No Cost Extension letter, we seek permission to abandon Task 3c and redirect the funds allocated for Task 3c to offset the increased effort and costs associated with the increased sample sizes required in Tasks 2a, 3a, 3b, 4a, 4b, 4b.2 and 4c, as described elsewhere in this Progress Report.

Task 4. *Test the hypothesis that ALM, but not ZOL, induces sleep by facilitating the mechanisms that underlie the transition to normal sleep.*

4a. Effects of ALM and ZOL on sleep-active brain areas: Data collection and analysis completed; manuscript in preparation.

4b. BF adenosine (ADO) release in response to oral ALM and ZOL. Data collection and analysis completed; manuscript in preparation.

4c. BF adenosine (ADO) release in response to ALM and ZOL by dialysis.

4c-2. Effects of BF microinjections of ALM and ZOL on sleep/wake and neurotransmitter release in the cerebral cortex. Data collection ongoing.

Progress - Task 4a: We proposed to determine whether ALM or ZOL alter the expression of Fos in sleep-active neuronal populations. Completion of Task 4a was delayed as we underestimated the number of rats that were necessary to obtain statistical significance: we utilized 54 rats (including those used for pilot studies) instead of the 24 rats originally projected. In the last report, we reported that sleep induced by both ZOL and ALM is permissive for activation of sleep-active cortical nNOS neurons, but that neither compound activates these neurons when rats are kept awake. Data collection and analysis of these studies have been completed. A series of follow-up studies examining the interaction between sleep pressure and ALM or ZOL treatment on cortical nNOS activation and EEG delta power was also completed.

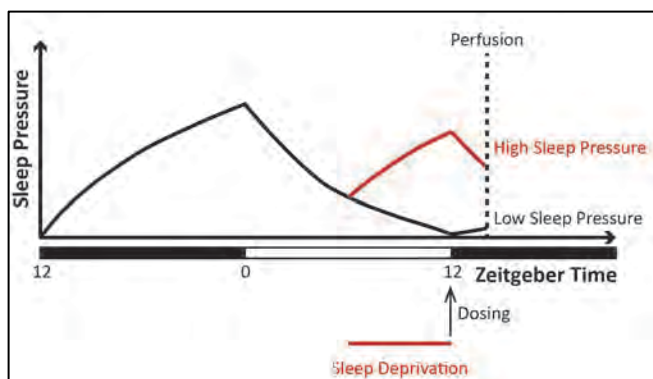


Figure 11. Experimental design. Sleep pressure is thought to increase during the active phase and decrease during the inactive phase (black curve). Thus, rats dosed at ZT12 have low sleep pressure. To increase sleep pressure but keep the circadian environment identical, some rats were subjected to 6 h of sleep deprivation starting at ZT6 (red curve). For histological experiments, rats were perfused 2 or 2.5 h after dosing. Black and white bars at bottom indicate light conditions.

Methods: To test the effects of pre-existing sleep pressure on efficacy of ZOL and ALM, four rats were administered ZOL (50 mg/kg), ALM (100 mg/kg), and VEH in a repeated measures design. A parallel set of four rats received a higher dose of the same drugs (100 mg/kg and 200 mg/kg, respectively). Each rat received each drug twice; once at ZT12, when sleep pressure is at its diurnal nadir. The other dose was given at the same Zeitgeber time but was preceded by 6 h of sleep deprivation, so that sleep pressure was increased (Figure 11).

To investigate the effect of sleep pressure on activation of sleep-active cortical neurons, rats were given ALM (100 mg/kg p.o.), ZOL (100 mg/kg p.o.) or VEH at ZT12, and either left

undisturbed (n=6 per drug condition) or subjected to sleep deprivation for the preceding 6 h (n=7 per drug condition; Figure 11). The rats were then left undisturbed for 2 h (2.5 h for ALM to offset the longer latency of the sleep-inducing effect) and then perfused. Brains were removed and processed for double immunohistochemistry for Fos and nNOS. Single labeled and double labeled nNOS neurons were counted in the cortex of coronal sections at three different levels for each rat.

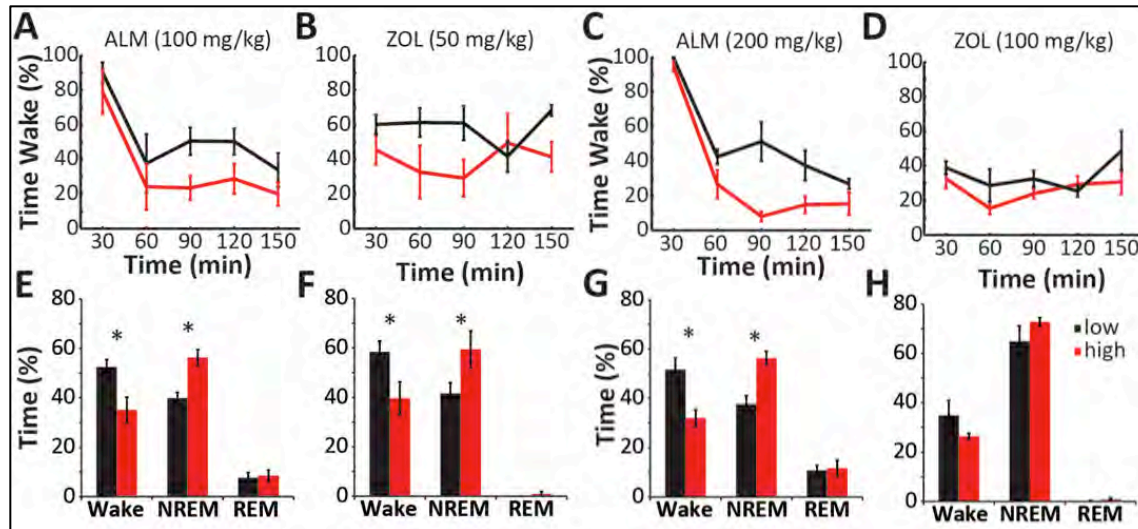


Figure 12. Effect of sleep pressure on efficacy of ZOL and ALM. Line graphs (A-D) depict wake time for the first 2.5 h following dosing; bar graphs (E-H) depict cumulative time spent in sleep states for the same time period. Black lines/bars indicate rats dosed at low sleep pressure; red lines/bars indicate rats dosed at high sleep pressure. After dosing with 50 mg/kg ZOL, as well as both ALM doses tested, rats spent more time asleep in the high sleep pressure condition. 100 mg/kg ZOL induced the same amount of sleep in the low and high sleep pressure conditions. **A.** Percent wake time following 100 mg/kg ALM. **B.** Percent wake time following 50 mg/kg ZOL. **C.** Percent wake time following 200 mg/kg ALM. **D.** Percent wake time following 100 mg/kg ZOL. **E.** Cumulative sleep-wake time following 100 mg/kg ALM. **F.** Cumulative sleep-wake time following 50 mg/kg ZOL. **G.** Cumulative sleep-wake time following 200 mg/kg ALM. **H.** Cumulative sleep-wake time following 100 mg/kg ZOL. *, significant effect of sleep pressure condition.

Figure 12 shows that the efficacy of ALM and ZOL is differentially affected by sleep pressure. High sleep pressure decreased wake time for both drugs at low doses (Fig. 12A-B, E-F). Doubling the ALM dose did not further decrease wake time for the low sleep pressure condition, but slightly decreased wake time for the high sleep pressure conditions, so that the difference between the conditions persisted (Fig. 12C, G). By contrast, doubling the ZOL dose further decreased wake time disproportionately more for the low sleep pressure than the high sleep pressure condition, so that the difference between the conditions disappeared (Fig. 12D, H).

For all three drug conditions, activation of cortical nNOS neurons was largely elevated by sleep pressure (Figure 13C-D, G-H). The time spent asleep in the low sleep pressure condition after dosing with ALM or ZOL was as high as in the high sleep pressure condition after VEH dosing (Figure 13A, B, E). Nonetheless, the percent of Fos-expressing cortical nNOS neurons was significantly lower (Figure 13F), indicating that sleep pressure is needed for activation of these neurons.

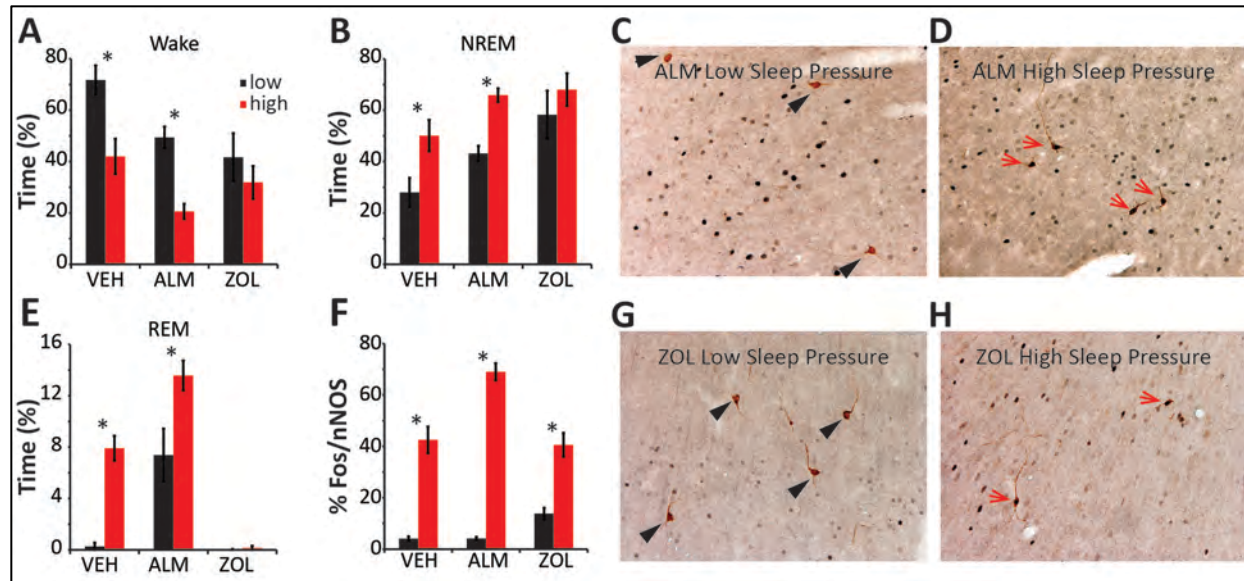


Figure 13. Time spent asleep and activation of nNOS neurons. **A**, **B**, and **E** depict time spent in the three sleep states during the 1.5 h preceding perfusion. **F**. Percentage of cortical nNOS neurons that were found to express Fos. **C** and **D**. Example micrographs of Fos/nNOS histochemistry after ALM dosing. Black arrowheads indicate single-labeled nNOS neurons, red arrows indicate double-labeled Fos/nNOS neurons. **G** and **H**. Example micrographs of Fos/nNOS histochemistry after ZOL dosing.

Sleep pressure is correlated with EEG delta power during NREM sleep. To determine whether sleep pressure-related regulation of the EEG is affected by ALM or ZOL, we calculated the EEG power spectra for each state during the 1.5 h before perfusion. As expected, ZOL significantly altered the wake (Figure 14E) as well as the NREM (Figure 14F) EEG power spectra compared to VEH treatment (Figure 14A-B), whereas the EEG spectra after dosing with ALM (Figure 14C, D) looked comparable to those of rats dosed with VEH. Strikingly, the sleep-pressure induced increase of NREM delta power (0.5-4.5 Hz) was present after ALM (Figure 14D) but not after ZOL dosing (Figure 14F). We conclude that ZOL interferes with physiological sleep pressure-related regulation of NREM delta power, whereas this process appears to be unaffected by ALM.

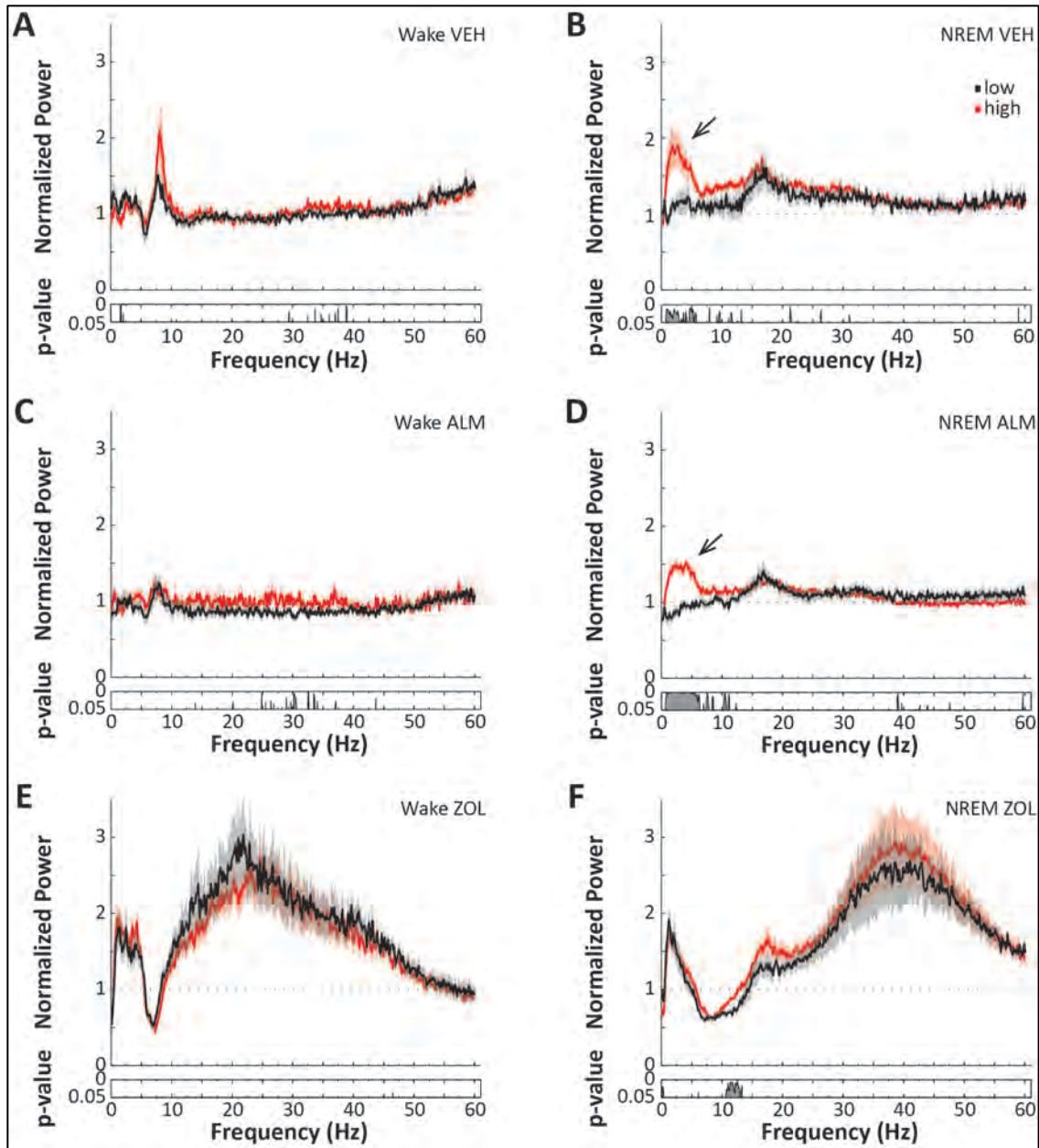
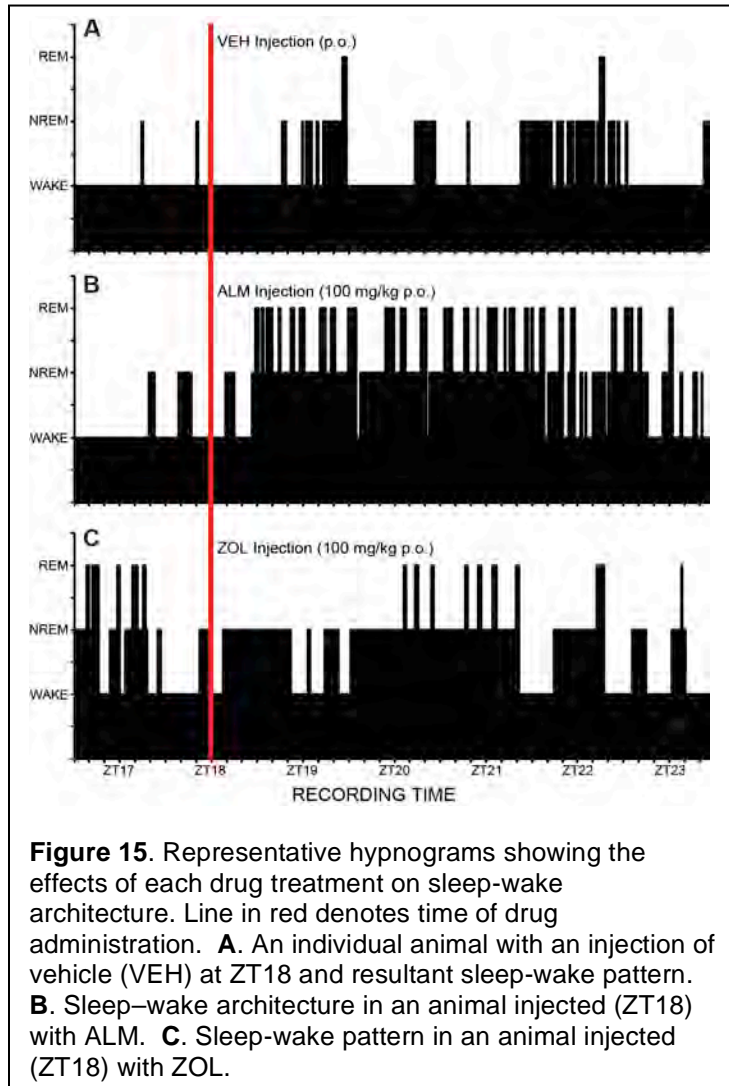


Figure 14. EEG power spectra of rats treated with VEH, ALM or ZOL. Spectra were normalized by the respective spectra of a 6h baseline recording (ZT1-6). Left column depicts wake spectra, right column depicts NREM spectra. REM spectra are not shown because for some conditions, REM sleep was too sparse to yield sufficient data. The spectra after VEH and ALM dosing are comparable (A-D). In contrast, EEG spectra show characteristic changes after ZOL dosing (E, F). Arrows highlight the sleep pressure-induced increase in NREM delta power after VEH and ALM dosing (B, D).

Progress Year 4 – Task 4b: We report here the combined results of all animals used for the first part of Task 4b, a study designed to examine the effects of oral ALM and ZOL on basal forebrain (BF) levels of adenosine (ADO), glutamate (GLU) and γ -aminobutyric acid (GABA) across the sleep-wake cycle. We tested the hypothesis that oral ALM induces sleep by facilitating the mechanisms that underlie the transition to normal sleep. In contrast to ZOL, which affects GABA_A receptors that are widely distributed in the CNS, we hypothesize that ALM acts through blockade of post-synaptic Hcrtr receptors, thereby disfacilitating excitation in the BF. We used *in vivo* microdialysis and HPLC analyses to examine BF GLU, GABA, and ADO efflux following oral ZOL (100 mg/kg), ALM (100 mg/kg), or placebo (VEH) combined with behavioral sleep analyses. Task 4b required use of 20 rats instead of the 16 originally planned.

Experimental Design: Male Sprague-Dawley rats (300±25 g; n=20) were housed in an ambient-controlled recording room under a 12 h light/12 h dark cycle (lights off at 04:00) with food and water available *ad libitum*. Room temperature (24±2°C), humidity (50±20% relative humidity), and lighting conditions were monitored continuously via computer. Animals were inspected daily in accordance with AAALAC and SRI guidelines. All animals were implanted with chronic recording devices (F40-EET, Data Sciences Inc., St Paul, MN) for continuous recordings of electroencephalograph (EEG), electromyograph (EMG), core body temperature (T_{core}), and LMA via telemetry as described previously (Morairty et al., 2008). Rats were then placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA) for surgical implantation of a unilateral, stainless steel 26-gauge guide cannula aimed at the BF for microdialysis recovery of ADO, GLU, and GABA. BF coordinates relative to bregma were P -0.3, L +2.0, V -5.0 (Paxinos and Watson, 2009). The electrodes, guide cannula (to permit microdialysis probe placement in the brain of freely-moving rats) and a stainless steel skull screw were fixed to the skull using dental cement. A dummy probe was inserted into the cannula to prevent occlusion prior to the onset of dialysis. For microdialysis, the dummy probe was removed and the microdialysis probe was inserted and locked into position such that the tip of the probe membrane extended 2.0 mm below the edge of the guide cannula.

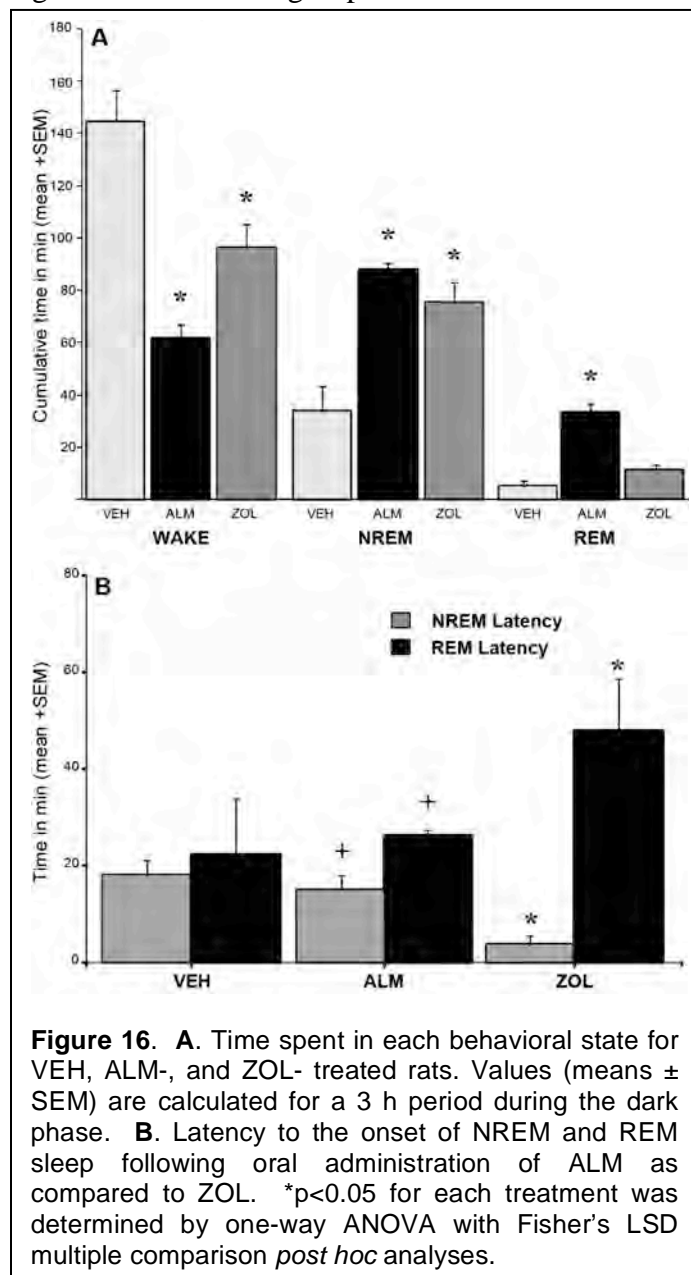


the BF. Drug doses included ALM (100 mg/kg), ZOL (100 mg/kg) and VEH. One of three drugs was subsequently given p.o. to the animals 6h into the dark period at ZT18 (the rats' normal active period), and eleven 30 min samples were collected over the subsequent 5.5 h to assess the effects of the drug on behavior and neurotransmitter release in the BF. Behavioral measures were simultaneously collected during the 5.5 h post-microdialysis. All samples were collected at 4°C and immediately stored at -80°C until processed for ADO by HPLC/UV and for GLU/GABA by HPLC-EC detection.

Behavioral Data Analyses: Following completion of data collection, sleep-wakefulness was scored in 10 s epochs by examining the recordings visually using Neuroscore software (Data Sciences Inc., St Paul, MN). Any epochs that contained recording artifacts were tagged and excluded from subsequent analyses. EEG and EMG data were scored for waking (W), rapid eye movement sleep (REM), and non-REM (NR). Individual state data were analyzed as time spent in each state (W, REM, and NR) per hour. Latency to NR and REM onset for each rat was calculated from the time of drug injection. To assess any pharmacological effects on the consolidation of behavioral states, cumulative time spent in W, NR, and REM and the duration

All animals were given a 2-3 wk post-surgical recovery period prior to entering the experimental paradigm, conditioned to the microdialysis chamber, and handled for at least 30 min every day for 1 wk prior to the onset of the experiment to limit stress on the day of the experiment from exposure to a novel environment. Animals were also given two separate 1 ml doses of vehicle on two days at least one week before the first experimental day. A microdialysis probe was inserted through the guide cannula 16 h prior to the onset of the experimental day and continuously perfused with aCSF. At the start of the experiment (4.5 hours into the dark period, ZT16.5), three 30 min baseline samples (1 µL/min flow rate, 30 µL total) were collected from freely-moving animals to assess basal levels of ADO, glutamate, and GABA and baseline EEG, EMG, T_b and LMA were collected to assess behavior. Telemetry data were recorded using DQ ART 3.1 software (Data Sciences Inc., St Paul, MN). Each rat received one treatment in random order (washout period minimum 1 week) with parallel microdialysis sampling of

and number of bouts of each state was calculated for 5.5 h following drug administration relative to each 30 min dialysis sample obtained pre- and post-drug administration. Descriptive statistics and analysis of variance (ANOVA) analyses were performed on all behavioral measures. Where ANOVA indicated a probability (*P*) value < 0.05, Dunnett's *post hoc* was used to determine significance between groups.

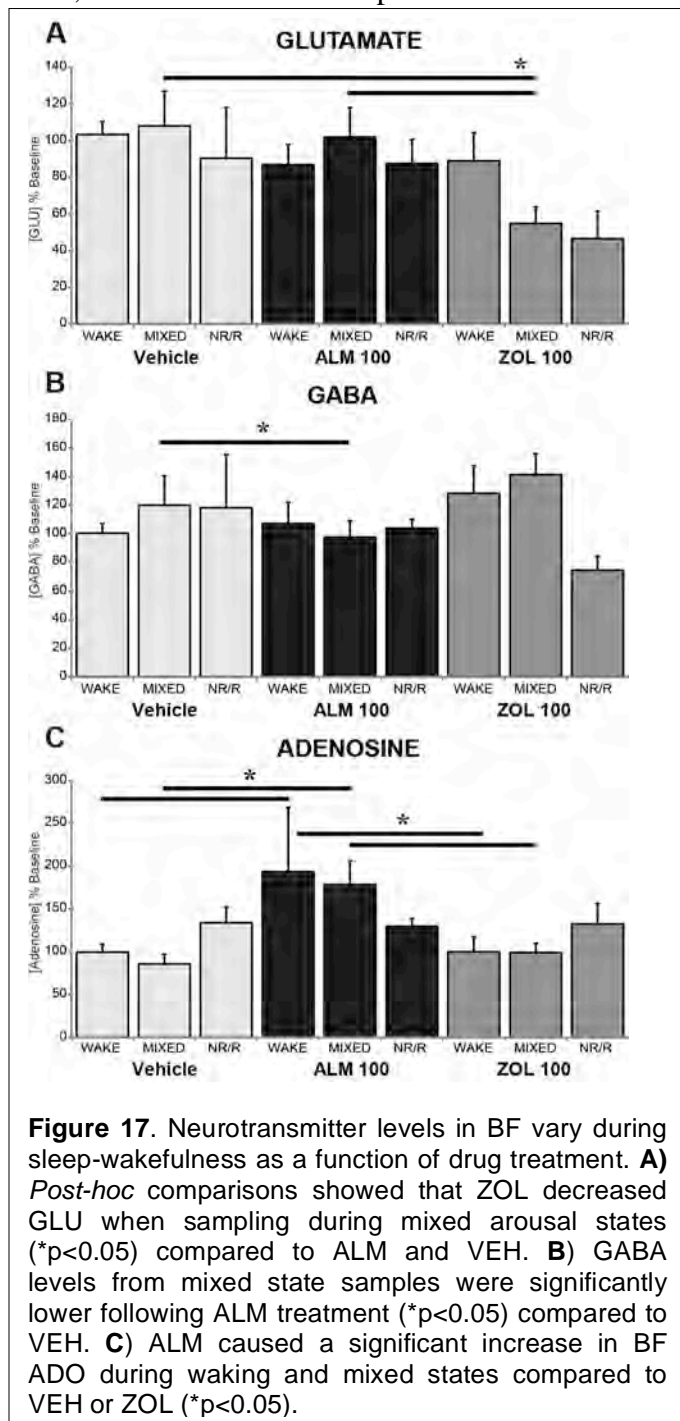


Newman-Keuls multiple pairwise comparison tests. A probability (*P*) value < 0.05 was used to evaluate the significance of all statistical tests.

Behavioral State Results. As illustrated in Figure 15, sleep-wake architecture varied significantly as a function of drug administration. Hypnograms show the effects of VEH, ALM (100 mg/kg, p.o.), and ZOL (100 mg/kg, p.o.) on the rats' sleep-wake cycle for each treatment condition. Figure 16 shows the cumulative time spent in the waking, NREM, and REM sleep

HPLC Analyses: All microdialysis samples were split (10 µL for ADO, 20 µL for AA/GABA) into two vials for HPLC analyses. ADO samples were separated by reverse-phase HPLC with a Kinetic column (Phenomenex C18 150 x 4.6mm) and monitored at 254 nm by UV. The mobile phase consisted of 10 mM Na₂HPO₄ (pH = 4.5), and 7% acetonitrile and was set to a flow rate of 0.8 mL/min. Calibration curves were constructed using Chromeleon 6.8.0 software (Dionex, Corp). Amino acids, glutamate and GABA were assayed using HPLC-EC. The mobile phase consisted of 100 mM Na₂HPO₄, 22% MEOH, and 3.5% acetonitrile, pH 6.75 and set to a flow rate of 0.4 mL/min. The amino acids were detected by precolumn derivitization using O-phthalaldehyde (OPA) and 2-mercaptoethanol (βME) with automation at 4°C, 2 min prior to injection into the HPLC. Separation was achieved with a reversed-phase column by Shiseido (Capcell Pak C18, 3.0 mm ID x 75 mm, 3 µm) and electrically detected at the following potentials; E1; +150 mV, E2; +550 mV, Guard +600 mV. Calibration curves were constructed using Chromeleon 6.8.0 software (Dionex Corp). Descriptive statistics and a two-way ANOVA were used to determine the effect of sleep-wake states on ADO, GLU, and GABA levels. Post hoc comparisons were performed using

states following delivery of either VEH, ALM (100 mg/kg p.o.), or ZOL (100 mg/kg p.o.). ANOVA revealed a significant drug effect on wake, NR, and R states (* $p < 0.05$). *Post hoc* analyses showed that ZOL and ALM had significant effects on the total amount of time spent in Wake, NREM and REM compared to VEH and on the latency to NREM and REM sleep.



Neurotransmitter Analyses and

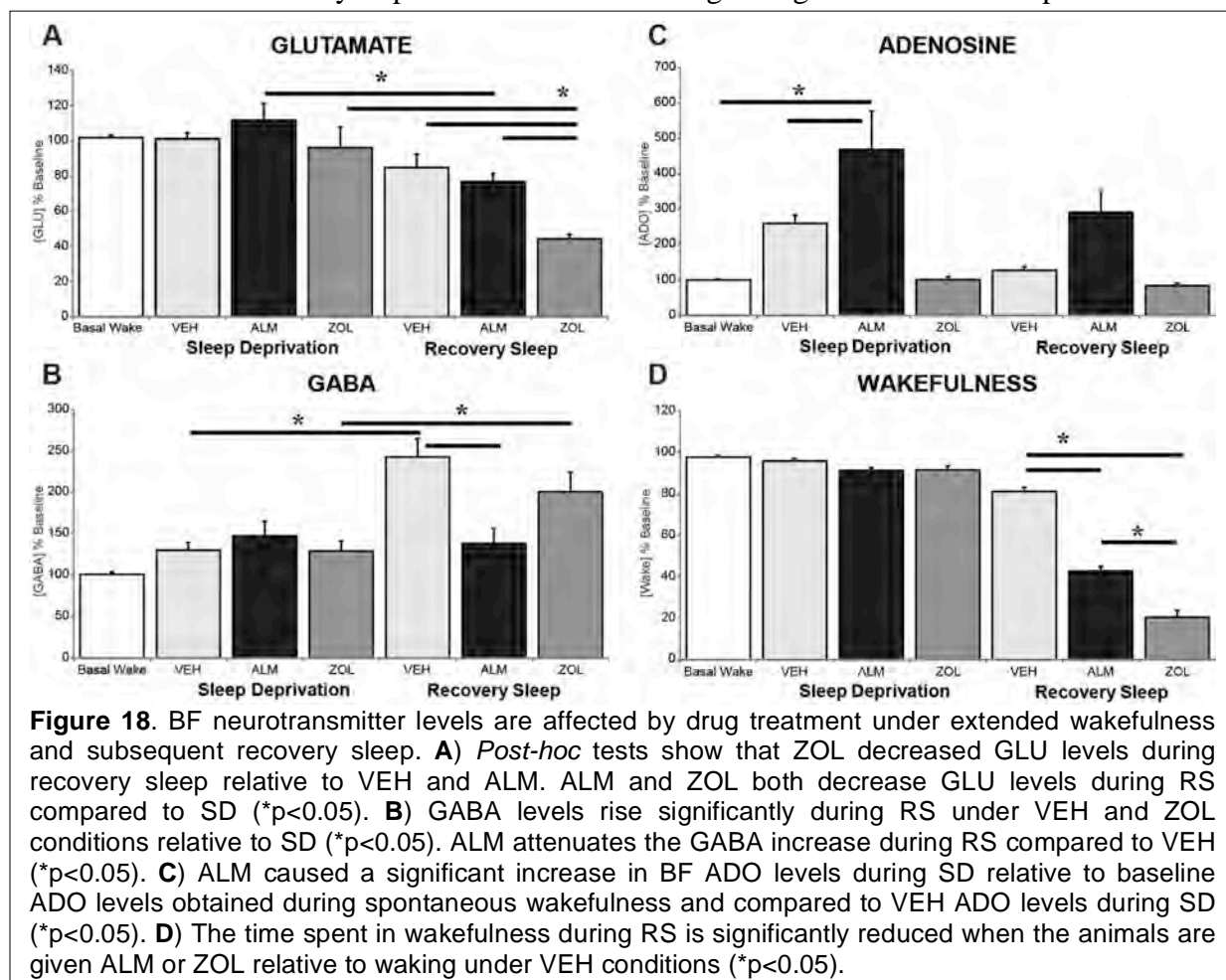
Results: Dialysis samples were split into two and processed for both ADO and GLU/GABA content. Two-way ANOVA revealed a significant drug x state interaction for all neurotransmitters. Newman-Keul's *post hoc* comparisons showed that oral ZOL (* $p < 0.05$) caused a significant decrease in BF glutamate levels (Figure 17A) when sampling during 30 min timeframes comprised of "mixed" arousal (MIXED) states (Wake, NREM, and REM) compared to ALM or VEH. There is an overall trend of decreased GLU levels across states of WAKE, MIXED, and timeframe comprised primarily of NREM and REM sleep (NR/R) when oral ZOL is administered to the animals, however these results do not achieve significance. Oral ALM concurrently decreased BF GABA levels (* $p < 0.05$) during dialysis sampling periods when the animals presented MIXED states (Figure 17B) relative to vehicle administration. Again, there is a trend toward decreased GABA levels during timeframes comprised of NR/R in the presence of ZOL, however these results do not achieve significance. Analyses of ADO levels revealed that oral ALM caused a significant increase in BF ADO (Figure 17C) during timeframes (30 min) comprised of WAKE and MIXED states compared to VEH (* $p < 0.05$). Administration of oral ALM additionally varies relative to oral ZOL effects on BF ADO (Figure 17C; * $p < 0.05$) under sampling conditions of WAKE and MIXED states. ALM

significantly promotes the amount of time spent in NREM and REM sleep as has been previously described (Dugovic et al., 2009). These data show that ALM significantly alters the latency to the onset of NREM and REM sleep compared to ZOL and VEH. During "mixed"

states that involve transitions between wake and sleep, ZOL attenuates GLU levels in the BF, whereas ALM does not. ALM appears to attenuate GABA levels during transitional mixed bouts of waking and sleep. Interestingly, ALM promotes and enhances ADO in the BF during wakefulness and mixed states, whereas ZOL does not alter ADO. During predominantly NREM/REM sleep, ZOL shows a trend toward attenuating GLU and GABA in the BF. These neurotransmitter results provide additional evidence for dynamic neurochemical changes underlying Hcrt modulation of sleep-wakefulness.

Progress Year 4 – Task 4b.2: In last year's progress report, we provided preliminary data from animals subjected to sleep deprivation to evaluate how a dual hypocretin antagonist (ALM) and a GABAergic agonist (ZOL) would alter neurotransmitter release under conditions of extended wakefulness. We report here the combined results of all 24 rats used for the second part of Task 4b (4b.2).

Experimental Design: Sprague-Dawley rats (males; n=24) were implanted with chronic recording devices and cannulae directed at the BF as described previously. Animals were given a 2-3 wk post-surgical recovery period prior to entering the experimental paradigm, conditioned to the microdialysis chamber, and handled for at least 30 min every day for 1 wk prior to the onset of the experiment to limit stress on the day of the experiment from exposure to a novel environment. A microdialysis probe was inserted through the guide cannula 16 h prior to the



onset of the experiment day and continuously perfused with aCSF. We performed 6 h of sleep deprivation (SD or extended wakefulness) and permitted 2 h of recovery sleep (RS). At the start of the experiment (4.5 hours into the dark period, ZT16.5), four 30 min baseline samples (1 μ L/min flow rate, 30 μ L total) were collected from freely-moving animals to assess basal levels of ADO, GLU, and GABA and baseline EEG, EMG, were collected to assess constant wakefulness. Each rat received one treatment in random order (washout period minimum 1 week) with parallel microdialysis sampling of the BF. Drug doses included ALM (100 mg/kg), ZOL (100 mg/kg) and VEH. One of three drugs was administered (p.o.) to the animals 6 h into the dark period (ZT18), and nine 30 min samples were collected to assess the effects of the drug on constant wakefulness and neurotransmitter release in the BF. Additionally, four samples were collected for an additional 2 h and the rats were allowed recovery sleep. All samples were collected at 4°C and immediately stored at -80°C until analysis by HPLC.

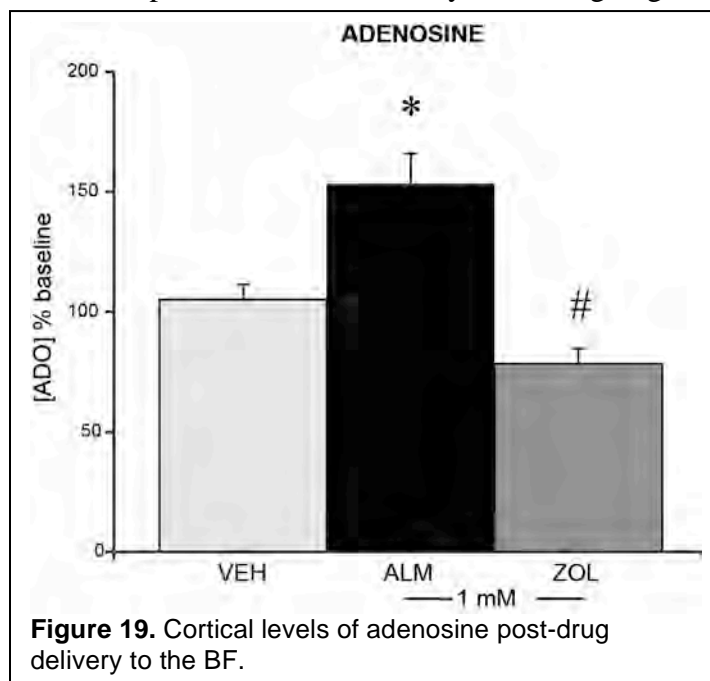
Behavior and Neurotransmitter Results: Figure 18 summarizes the effects of VEH, ZOL, and ALM administration (p.o.) in animals subjected to 6 h of sleep deprivation and the first 2 h of recovery sleep for each neurotransmitter system following drug delivery. Analyses of neurotransmitter levels demonstrate that BF neurotransmitter levels are affected by drug treatment delivered during sleep deprivation and persist into recovery sleep. As shown in Figure 18A, ZOL caused a significant drop in GLU levels during recovery sleep relative to VEH or ALM. ALM and ZOL both attenuate RS GLU levels relative to their SD counterparts (* $p < 0.05$). On the other hand, ZOL enhances GABA levels during RS (Figure 18B), similar to the rise in GABA under VEH conditions and above GABA levels during SD ZOL administration (* $p < 0.05$). Figure 18B shows that ALM attenuates the GABA increase during RS compared to VEH (* $p < 0.05$). Interestingly, ALM promotes a significant increase in BF ADO levels during sleep deprivation (Figure 18C) relative to baseline ADO levels obtained during spontaneous wakefulness and when animals receive VEH during SD (* $p < 0.05$). Figure 18D highlights a concomitant decrease in the time spent awake during RS when the animals receive either ALM or ZOL relative to VEH (* $p < 0.05$). These results suggest that these neurotransmitter systems are affected by sleep-promoting drugs when sleep deprived and can persist into recovery sleep. The effects of ALM on ADO in the BF during spontaneous waking and during extended wakefulness are similar to previously published data demonstrating that spontaneous BF ADO levels progressively increase during sleep deprivation and decrease during recovery sleep (Porkka-Heiskanen et al., 2000), suggesting that ALM may act permissively to enhance adenosine in addition to modulating the Hcrt system and promote sleep. These neurotransmitter results provide additional evidence for dynamic neurochemical changes underlying Hcrt modulation of sleep-wakefulness. A manuscript is currently in preparation and all of the behavioral and neurotransmitter analyses for the studies performed under Task 4b and 4b.2 will be submitted for publication this year.

Progress Year 4 – Task 4c: The originally proposed study design for Task 4c (BF ADO release in response ALM and ZOL by local dialysis) was deemed not feasible as neither ALM nor ZOL readily pass across the dialysis membrane (testing in-house). As previously stated in last year's Progress Report, we proposed to microinject ALM and ZOL into the BF and collect microdialysis samples from the prefrontal cortex (a nNOS sleep-cortical region as discovered by Gerashchenko et al, 2008) of freely-moving animals and assess neurotransmitter levels, along with simultaneous EEG and EMG behavioral measures. We proposed that 70 rats would be required to reach statistical power for the new Task 4c: a minimum of 10 animals per drug

condition, with 3 drug concentrations per drug (e.g., low, middle, and high dose) for ALM and ZOL compared to SAL. To date, no studies have reported the effects of central microinjections of ALM or ZOL and its effect on behavior or transmitter levels in brain.

Experimental Design: Sprague-Dawley rats (males; $n=3$) were implanted with chronic recording devices and three cannulae: bilateral cannulae mounted over the skull and directed at the BF with the same coordinates as described previously and a third dialysis cannula directed to the prefrontal cortex (PFCx). PFCx coordinates relative to bregma were P +3.2, L +0.6, V -2.0 (Paxinos and Watson, 2009). Animals were given a 2-3 wk post-surgical recovery period prior to entering the experimental paradigm, conditioned to the microdialysis chamber, and handled for at least 30 min every day for 1 wk prior to the onset of the experiment to limit stress on the day of the experiment from exposure to a novel environment. A microdialysis probe was inserted through the guide cannula 16 h prior to the onset of the experiment day and continuously perfused with aCSF. At the start of the experiment (4.5 hours into the dark period, ZT16.5), three 30 min baseline samples (1 $\mu\text{L}/\text{min}$ flow rate, 30 μL total) were collected from freely-moving animals to assess neurotransmitter content with simultaneous sleep-wake data collection via telemetry. Each rat received one microinjected drug dose in random order (washout period minimum 1 week) with parallel microdialysis sampling of the PFCx. One of three drugs was delivered through the dual BF cannula to the animals 6 h into the dark period at ZT18, and 12 additional 30 min samples were collected to assess the effects of the microinjected drug on behavior and neurotransmitter release in the BF.

Neurotransmitter Results: Here we show preliminary data from 3 rats at one dose of ALM and ZOL. To date, we have examined cortical ADO levels following BF microinjections of ZOL (1 mM; 0.6 $\mu\text{g}/0.2 \mu\text{L}$), ALM (1 mM; 1.0 $\mu\text{g}/0.2 \mu\text{L}$), or VEH (in aCSF) combined with behavioral analyses. Preliminary analyses revealed a significant main effect of drug on ADO levels. Sleep-wake behavioral analyses are ongoing. *Post hoc* comparisons showed that ALM



microinjected into the BF ($n=3$ rats; * $p<0.05$) caused a significant increase in cortical ADO that lasts up to 6 h post microinjection compared to VEH control (Figure 19). Conversely, administration of ZOL ($n=3$) to the BF significantly decreased cortical ADO levels (# $p<0.05$) compared to VEH and ALM. These results provide novel evidence suggesting that there are central and peripheral effects (as shown in the results of Task 4b) of sleep-promoting drugs on the behavioral neurochemistry underlying Hcrt modulation of sleep-wakefulness.

Task 6: Utilize optogenetics and *in vivo* physiology to compare the neural circuitry underlying ALM-induced vs. ZOL-induced sleep.

- 6a. Determine whether activation of the Hcrt system is sufficient to induce arousal in the presence of ALM vs. ZOL.
- 6b. Determine whether ALM affects the activity of subcortical sites downstream from the Hcrt neurons.
- 6c. Determine how ALM and ZOL affect the activity of cortical neurons.

Progress - Task 6a: To replicate active Hcrt neuronal circuitry and test the effects of ALM and ZOL on behavior and synaptic activity using *in vivo* and *in vitro* protocols respectively, we established a colony of *orexin-tTA; Tet-O ChR2(C128S)* mice (gift from Dr Akihiro Yamanaka, Nagoya University). Under the direction of the HCRT promoter, these mice contain a modified channelrhodopsin-2 (ChR2), a light-gated cation channel, and the fluorescent marker GFP. This construct design ensures selective expression of ChR2 directed to Hcrt neurons within the hypothalamus, enabling specific targeting of the Hcrt circuitry. The modified ChR2, a step-function opsin (SFO), has subtle structural changes which, when activated by blue light application either through the microscope *in vitro* or through optical fibers implanted *in vivo*, depolarizes the neuron of interest by evoking an inward current. Compared to traditional ChR2s, this SFO-ChR2 remains in the open state longer to prolong this response, increasing the likelihood excitatory inputs will cause the cell to fire action potentials. This SFO-ChR2 can also be closed prematurely by yellow light illumination, allowing for controlled temporal precision (<2 ms) and fine electrical command over Hcrt neuronal activity. To address Task 6a, we initially validated this mouse model for *in vivo* and *in vitro* purposes.

Methods: To validate expression of SFO-ChR2 to Hcrt neurons, immunohistochemical protocols for Hcrt-A, Hcrt-B and GFP (marker for SFO-ChR2) were performed on 4% paraformaldehyde-fixed sagittal brain sections from *orexin-tTA; Tet-O ChR2(C128S)* mice (n=4). To test the functionality of SFO-ChR2 in this model, we used *in vitro* electrophysiology on coronal hypothalamic slices and recorded neuronal activation in cell-attached and whole-cell configurations. Hcrt neurons were located using GFP fluorescence and confirmed as Hcrt cells on the basis of electrophysiological recordings. Blue and yellow light pulses to drive SFO-ChR2-evoked Hcrt activity were delivered via computer-controlled pulse trains.

In vivo behavioral validation was performed by implanting EEG/EMG leads for tethered recording of sleep-wake state. To stimulate SFO-ChR2-expressing Hcrt neurons, a 0.5 mm fiber optic was inserted within 1mm of the main lateral hypothalamic Hcrt field. After a 3 week post-surgical recovery period, baseline recording (48 h) was performed and then a light-pulse protocol applied in the middle of the light phase (ZT6).

Following analysis and refinement of the pulse train protocol, determined by *in vitro* and *in vivo* pilot studies (presented herein), we will then repeat the experimental paradigm in the presence of vehicle, ZOL (50 mg/kg) or ALM (100 mg/kg), all administered IP and at different circadian time-points to assess the efficacy of these drugs in relation to Hcrt activity.

Preliminary results: Immunohistochemistry revealed 81.2 ± 2.98 % of Hcrt-containing neurons colocalized with GFP, indicating that these cells expressed SFO-ChR2. The predominant population of Hcrt neurons containing GFP were those identified with Hcrt-A compared to Hcrt-B ($68 \pm 8.2\%$ vs $50 \pm 12.1\%$, n=4 mice), although there is significant overlap between both peptide markers. Figure 20A illustrates Hcrt-containing cells identified with antibodies for GFP (green) and Hcrt-A (yellow).

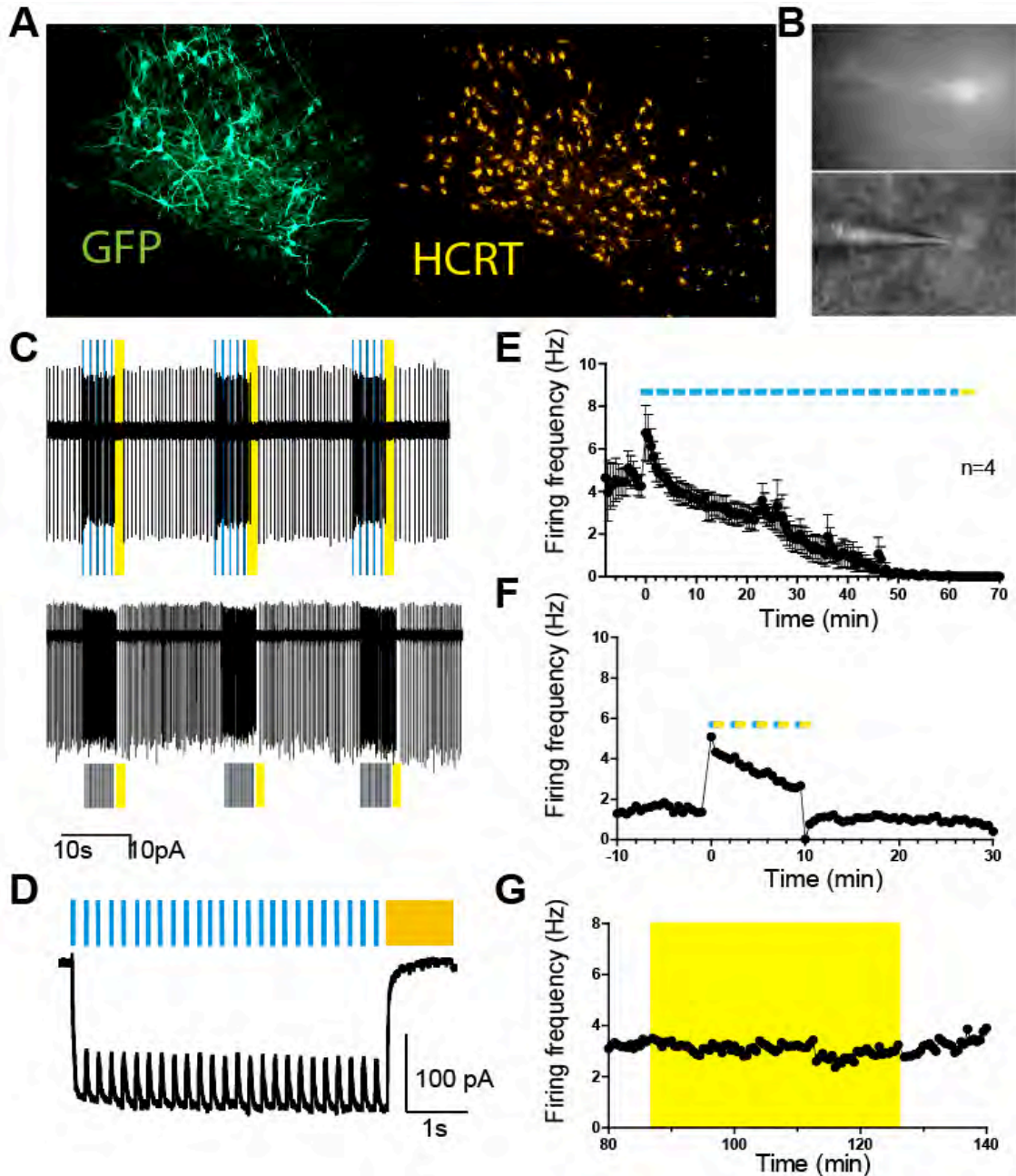


Figure 20. *In vitro* optogenetic stimulation of Hcrt neurons. **A.** Immunohistochemical detection of SFO-ChR2 (GFP, green) and Hcrt-containing neurons (Hcrt-A, yellow) reveal 68% of Hcrt neurons express SFO-ChR2. **B.** GFP attached to the SFO-ChR2 construct enables the fluorescent detection of Hcrt neurons (*upper*) that were then selectively patched (*lower*) for recording. **C.** Different frequency pulse trains of light stimulation were tested (1Hz, *upper*, 5Hz, *lower*) to ensure temporal precision could be achieved. **D.** Inward current induced by activation of SFO-ChR2 during a 5 Hz, 25 ms pulse train of blue light. Current is terminated by brief application of yellow light which closes the channel. **E.** In cell-attached mode, sustained 2 Hz, 24 ms blue-light stimulation resulted in an initial increase in firing frequency which then dramatically reduced, indicating either depolarization block or a desensitization of the SFO-ChR2 to sustained blue light ($n=4$). **F.** When the blue light train stimulation was modified to contain 1s yellow every 3rd pulse, a more sustained increase in firing rate was achieved. **G.** Responses of neurons to blue light stimulation protocols was due to SFO-ChR2 activation and not a heating artifact as the same results were not achieved when yellow light was used.

After demonstrating successful expression of the SFO-ChR2 in Hcrt neurons, the functional competence of this model was confirmed *in vitro*. Native expression of the SFO-ChR2 was detected under the GFP filter (Figure 20B, *upper*) and the cell patched for electrical recording (Figure 20B, *lower*). Different pulse train protocols were applied (blue light at 1-5Hz for 5 s pulse train followed by 1 s yellow pulse over 1 min) under different recording configurations (Figure 20C, cell-attached mode). In voltage clamp, these pulse trains all resulted in an inward current during light-stimulation indicating SFO-ChR2 activation. The amplitude of this current varied across cells and is likely correlated to the SFO-ChR2 membrane expression. An example of a large response during a 5 Hz protocol is illustrated in Figure 20D.

In current-clamp recordings, membrane depolarization occurred during light-stimulation, although the peak change occurred after stimulation ceased, supporting the longer activation kinetics of SFO-ChR2 (data not shown). When a more prolonged stimulation train of only blue light was applied in cell-attached mode (2 Hz, 24 ms pulse train for 1 h), an initial increase in firing rate was observed that decreased over the remainder of the light stimulus (Figure 20E). This apparent reduction in firing rate could be due to depolarization block as the membrane potential cannot be ascertained in this mode or possibly due to a desensitization of the SFO-ChR2 to blue light. When the protocol was modified to interleave yellow light every 3 pulses of blue light (3 Hz 10 ms blue, 1 s yellow) for a 10 min train, the change in firing rate was more pronounced and sustained in the depolarization direction (Figure 20F). Thus, this type of 'interleaved' protocol may prove more effective for sustained Hcrt neuronal activation if desensitization of the channel is prone to occur during sustained stimulation trains. Moreover, the responses recorded are due to SFO-ChR2 activation and not attributable to a heating artifact caused by light stimulation since constant yellow light alone (used to inactivate the channel) did not affect the basal firing rate of Hcrt cells (Figure 20G).

For behavioral characterization, we have optimized the surgical technique required for successful EEG/EMG and optical fiber implantation. In addition, we have tested the patency of this system for prolonged light stimulation and simultaneous EEG recording. After a 3 week post-surgical recovery period, the implanted mouse behaved normally and exhibited normal EEG/EMG signatures, including normal transitions between behavioral states (Figure 21A-B). Blue light stimulation (white bar) to activate the SFO-ChR2 (2 Hz 40 ms pulse train for 1 h) at ZT6 induced a prolonged wakefulness within a few minutes of pulse train onset (Figure 21C-D). Mean wake bout duration increased nearly 6-fold and the time spent awake increased more than 3-fold when compared to the same circadian time-point during baseline recording (Figure 21E-F).

Progress - Task 6b: Terminal stimulation protocols are being designed to replicate Hcrt release at subcortical sites downstream from the Hcrt neurons. Of interest are the histaminergic tuberomammillary nucleus (TMN) and the norephinergeric locus coeruleus (LC), as these regions receive substantial regulation by Hcrt circuitry and may thus be key sites for ALM action.

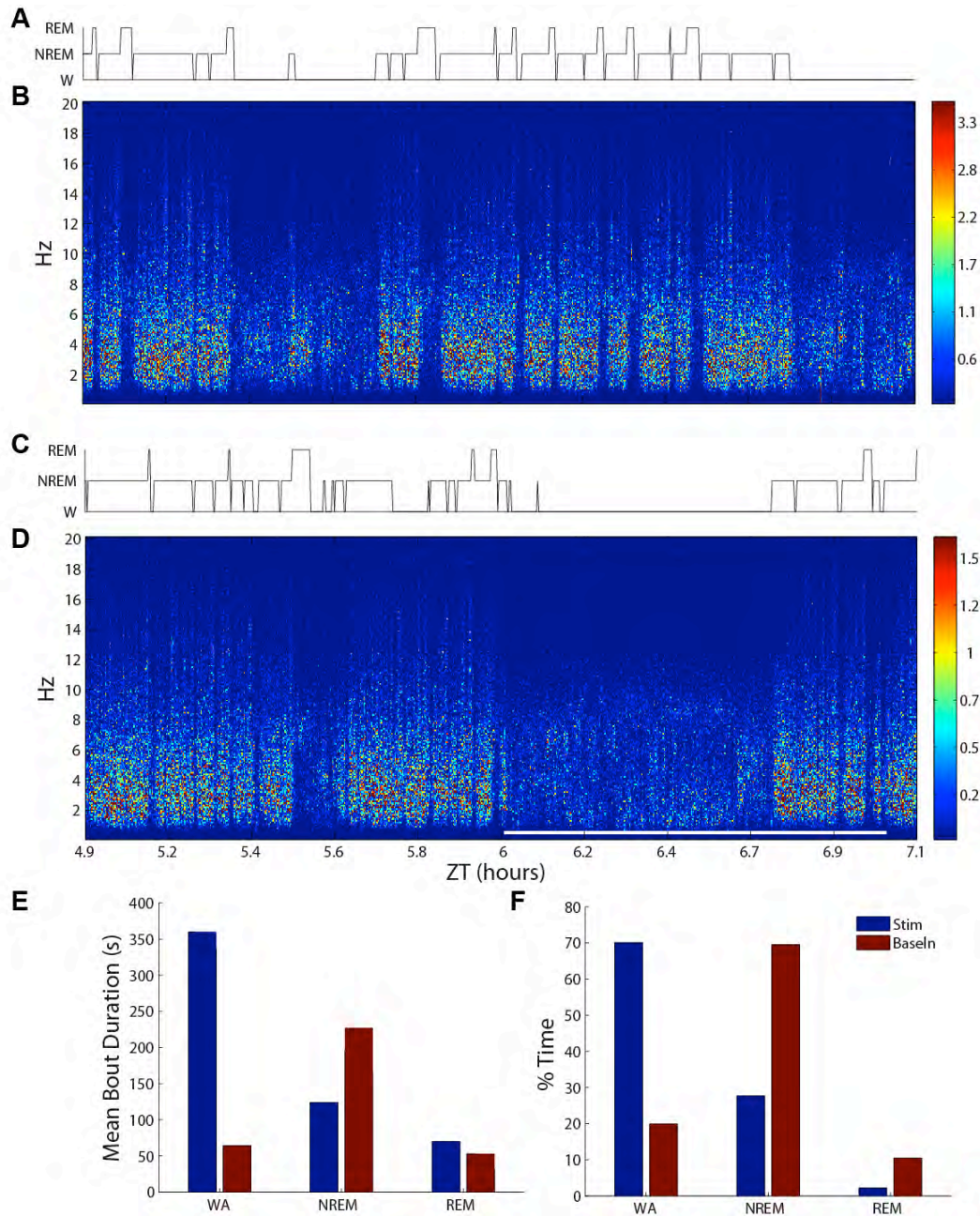


Figure 21. *In vivo* bilateral optogenetic stimulation of Hcrt neurons. **A.** Hypnogram demonstrating sleep-wake behavior of an implanted mouse during baseline recording (ZT 4.9 and 7.1). **B.** Power spectra of the EEG recording during baseline. **C.** Hypnogram of the same animal recorded one week later at the same circadian time in which bilateral optical stimulation with pulses of blue light were applied between ZT6 and ZT7. **D.** Power spectrum before and during optical stimulation. **E.** Mean bout duration and **F,** percentage of time in each state during optogenetic stimulation (Stim) and baseline (baseln) between ZT6 and ZT7.

Plans for Year 5:

Tasks 2a and 2b: Manuscript to be submitted for publication in *Frontiers in Neuropharmacology* by 8/31/13.

Task 2c: Data analysis for the rPVT study to be completed and manuscript to be submitted for publication. Results to be presented at the Society for Neuroscience meeting in San Diego in November, 2013.

Task 3a: We will complete processing and analysis of Fos studies in early 2014. Manuscript will be submitted for publication by mid-2014.

Task 3b: Data collection for the third and final lesion study to be completed by 8/31/13. Analyses for all three lesion studies is expected to be completed and a manuscript will be submitted for publication by 12/31/13.

Task 4a: A manuscript describing the effects of ALM and ZOL on sleep-active neurons will be submitted for publication by 9/30/13.

Task 4b: The behavioral and neurotransmitter analyses will be submitted for publication by 8/31/13.

Task 4c: We will continue collecting data from additional animals in order to reach our proposed statistical power of 10 rats per treatment group in the new Task 4c. Results to be presented at the Society for Neuroscience meeting in San Diego in November, 2013. The behavioral and neurotransmitter analyses will then be submitted for publication.

Task 6a: We will complete implantation of fiber optics and EEG/EMG leads in 8 male *orexin-tTA; Tet-O ChR2(C128S)* mice and study the changes in sleep architecture induced by optogenetic activation of Hcrt neurons after administration of vehicle, ALM and ZOL.

Tasks 6b and 6c: We will initiate and complete these Tasks in Year 5.

KEY RESEARCH ACCOMPLISHMENTS

- Completed analysis of EEG records for Task 2a that confirmed the hypnotic efficacy of the administered doses of ALM and ZOL (Figure 1).
- Completed data collection and analysis of all WM data needed for Task 2b that discerned significant differences between ALM and ZOL in the performance of the spatial working memory task (Figures 3-4).
- Completed the acquisition and setup of the rPVT system. Completed the in-life portion of the rPVT experiments in Task 2c. This included all conditions initially proposed (ALM and ZOL at 100 mg/kg, p.o., and VEH following both undisturbed and SD conditions) and 4 additional conditions (ALM and ZOL and 30 mg/kg, p.o., following both undisturbed and SD conditions). Since this was a repeated measures experiment, all rats underwent all conditions in a semi counter-balanced order. Analysis has been initiated on this data set.
- Completed processing and analysis of the effects of ZOL and ALM on Fos expression patterns following sleep deprivation for most of the animals described in Task 3a (Figures 5-7)
- Identified that histamine- and hypocretin-producing neurons are inhibited by ZOL but not ALM following sleep deprivation (Figures 6-7).

- Completed the number of animals needed for Task 3b.2 evaluating the effects of LC lesions on ALM vs. ZOL-induced sleep in the Sprague-Dawley rat (Figures 8-10).
- Completed experiments and analysis to compare effects of ZOL and ALM on sleep-active cortical neurons.
- Completed experiments to compare interactions of ZOL and ALM with sleep pressure (Figures 11-14).
- Completed the number of animals needed for Task 4b evaluating the effects of oral ALM vs. ZOL on neurotransmitter release in the Sprague-Dawley rat (Figures 15-17)
- Completed the number of animals needed for Task 4b.2 evaluating the effects of oral ALM vs. ZOL on animals under conditions of extended wakefulness and recovery sleep that was established as a complementary study under Task 4b (Figure 18).
- Began a preliminary cohort of animals needed for the new study in Task 4c, evaluating the behavioral and neurochemical effects of central, localized microinjections of ALM vs. ZOL in the BF and cortical neurotransmission (Figure 19).
- Established a healthy colony of transgenic *orexin-tTA; Tet-O ChR2(C128S)* mice needed for Task 6 and showed that they can be excited by blue light pulses (Fig. 20) and that *in vivo* optogenetic stimulation of Hcrt cells can cause changes in sleep architecture (Fig. 21).

REPORTABLE OUTCOMES

L. Dittrich, D. P. Warrier, A. J. Wilk, S. R. Morairty, T. S. Kilduff (2012). Non-REM sleep is permissive but sleep pressure is required for full activation of cortical nNOS neurons. Program No. 17.07. *2012 Neuroscience Meeting Planner*. New Orleans, LA: Society for Neuroscience, 2012. Online.

S. W. Black, S. R. Morairty, S. P. Fisher, T.-M. Chen, D. R. Warrier, T. S. Kilduff (2012). Almorexant promotes sleep and exacerbates cataplexy in a murine model of narcolepsy. Program No. 486.19. *2012 Neuroscience Meeting Planner*. New Orleans, LA: Society for Neuroscience, 2012. Online.

M. D. Schwartz, L. Dittrich, S. P. Fisher, W. Lincoln, H. Liu, M. A. Miller, D. R. Warrier, A. J. Wilk, S. R. Morairty, T. S. Kilduff (2012). Effects of a dual hypocretin receptor antagonist on sleep and wakefulness in rats. Program No. 799.23. *2012 Neuroscience Meeting Planner*. New Orleans, LA: Society for Neuroscience, 2012. Online.

S.R. Morairty, A. Wilk, W. Lincoln, L. Dittrich, T.C. Neylan, T.S. Kilduff (2013). The hypocretin receptor antagonist almorexant promotes sleep in rats and does not impair performance in spatial reference memory or spatial working memory tasks. *Sleep* 36 Abstract Supplement: A73-A74.

CONCLUSION

During Year 4, results continued to accumulate that are consistent with the hypothesis that disfacilitation of wake-promoting systems by the hypocretin (Hcrt) receptor antagonist almorexant (ALM) results in less functional impairment than the inhibition of neural activity produced by the benzodiazepine receptor agonist zolpidem (ZOL). In Year 4, the previously-reported results on spatial reference memory (Task 2a) and spatial working memory (Task 2b) were complemented by Psychomotor Vigilance Test studies (Task 2c). Collectively, these studies demonstrate that rodents treated with ALM were mostly indistinguishable from vehicle whereas impairments were clearly evident under ZOL. If this observation is confirmed in humans, it would have enormous implications for the management of disturbed sleep in both military and civilian populations. We also found that two wake-active neuronal populations, the histaminergic and Hcrt neurons, can be recruited in the presence of ALM after sleep deprivation but not in the presence of ZOL (Task 3a). Lesions of the locus coeruleus (LC), a wakefulness-promoting area, abolished the ALM-induced decrease in NREM sleep latency without affecting the ZOL-induced decrease (Task 3b). High sleep pressure, rather than the actions of ALM or ZOL *per se*, is critical for activation of sleep-active cortical neurons (Task 4a). ALM promoted adenosine release in the BF (Task 4b) and cortex (Task 4c), particularly during waking. Hcrt neurons expressing channelrhodopsin-2 can be excited by blue light pulses *in vitro* and preliminary *in vivo* experiments indicate that optogenetic activation of Hcrt cells can cause changes in sleep architecture (Tasks 6a). Together, these mechanistic studies in Tasks 3-6 provide insights into the neurobiological mechanisms that underlie the differential response to ALM and ZOL found in the behavioral studies conducted in Task 2.

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APPENDICES

L. Dittrich, D. P. Warrier, A. J. Wilk, S. R. Morairty, T. S. Kilduff (2012). Non-REM sleep is permissive but sleep pressure is required for full activation of cortical nNOS neurons. Program No. 17.07. *2012 Neuroscience Meeting Planner*. New Orleans, LA: Society for Neuroscience, 2012. Online.

S. W. Black, S. R. Morairty, S. P. Fisher, T.-M. Chen, D. R. Warrier, T. S. Kilduff (2012). Almorexant promotes sleep and exacerbates cataplexy in a murine model of narcolepsy. Program No. 486.19. *2012 Neuroscience Meeting Planner*. New Orleans, LA: Society for Neuroscience, 2012. Online.

M. D. Schwartz, L. Dittrich, S. P. Fisher, W. Lincoln, H. Liu, M. A. Miller, D. R. Warrier, A. J. Wilk, S. R. Morairty, T. S. Kilduff (2012). Effects of a dual hypocretin receptor antagonist on sleep and wakefulness in rats. Program No. 799.23. *2012 Neuroscience Meeting Planner*. New Orleans, LA: Society for Neuroscience, 2012. Online.

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Presentation Abstract

Program#/Poster#: 17.07

Presentation Title: [Non-REM sleep is permissive but sleep pressure is required for full activation of cortical nNOS neurons](#)

Location: 388

Presentation time: Saturday, Oct 13, 2012, 2:30 PM - 2:45 PM

Authors: ***L. DITTRICH**, D. P. WARRIER, A. J. WILK, S. R. MORAIRTY, T. S. KILDUFF;
Biosci., SRI Intl., Menlo Park, CA

Abstract: Cortical neurons immunoreactive for neuronal nitric oxide synthase (nNOS) are selectively activated during sleep. We have proposed that cortical nNOS neurons are inhibited by wake-promoting brain regions and activated by sleep-promoting factors that accumulate during prolonged wakefulness. To test this hypothesis, we compared nNOS neuron activation in animals that slept comparable amounts but under conditions of either high or low sleep pressure. To this end, we administered the GABA-A agonist zolpidem (ZOL, 100 mg/kg p.o., n=5) or vehicle (VEH, n=5) to Sprague Dawley rats at ZT12, when sleep pressure is naturally lowest. As a comparison, sleep pressure was increased by 6h of sleep deprivation before administering ZOL (n=6) or VEH (n=5) at ZT12. All 4 groups of rats were perfused 90-120 min after dosing (ZT13.5-14). To test whether ZOL was permissive for nNOS neuron activation in the absence of sleep, additional rats were administered ZOL (n=6) or VEH (n=7) at ZT18 and sleep deprived until perfusion 90-120 min later. EEG/EMG recordings for sleep/wake determination were performed for 8h prior to perfusion and a preceding 24h baseline period. The percentage of cortical nNOS neurons immunoreactive for c-FOS was determined in histological sections.

The ZOL-treated groups that were allowed to sleep did not differ in the time spent asleep in the 1.5h before perfusion. However, the ZOL-treated group with low sleep pressure showed a significantly lower proportion of c-FOS/nNOS neurons ($19.9\% \pm 0.05$ SEM) than either the ZOL-treated or the

VEH-treated groups with high sleep pressure (58.1 ± 0.05 , 44.2 ± 0.06 , respectively), indicating the importance of sleep pressure for activation of cortical nNOS neurons. The proportion of c-FOS/nNOS neurons in the rats treated with ZOL at ZT18 and not allowed to sleep did not differ from VEH control (6.1 ± 1.4 vs. 3.3 ± 0.8 , respectively), indicating that neither sleep pressure nor GABA-A agonism in the absence of sleep is sufficient for activation of cortical nNOS neurons.

Thus, in agreement with our hypothesis, the occurrence of sleep removes wake-related inhibition of cortical nNOS neurons but prior accumulation of sleep pressure is required for full activation of this neuronal population. These results support the concept of a role for nNOS neurons in sleep homeostasis, the physiological adaptation of increased sleep intensity or duration in response to elevated sleep pressure.

Disclosures: **L. Dittrich:** None. **D.P. Warriar:** None. **A.J. Wilk:** None. **S.R. Morairty:** None. **T.S. Kilduff:** None.

Keyword(s): SLEEP DEPRIVATION
SLEEP HOMEOSTASIS
NITRIC OXIDE SYNTHASE

Support: USAMRAA Award Number W81XWH-09-2-0081

DFG fellowship DI 1718/1-1

[Authors]. [Abstract Title]. Program No. XXX.XX. 2012 Neuroscience Meeting Planner. New Orleans, LA: Society for Neuroscience, 2012. Online.

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Presentation Abstract

Program#/Poster#: 486.19/VV7

Presentation Title: [Almorexant promotes sleep and exacerbates cataplexy in a murine model of narcolepsy](#)

Location: Hall F-J

Presentation time: Monday, Oct 15, 2012, 3:00 PM - 4:00 PM

Authors: ***S. W. BLACK**, S. R. MORAIRTY, S. P. FISHER, T.-M. CHEN, D. R. WARRIER, T. S. KILDUFF;
Biosci. Div., SRI Intl., MENLO PARK, CA

Abstract: Disruption of the hypocretin (Hcrt, also known as orexin) neuropeptide signaling system results in a narcoleptic phenotype characterized by excessive sleepiness, fragmented sleep, abnormally timed Rapid-Eye-Movement (REM) sleep, and cataplexy—an emotionally triggered, sudden loss of muscle tone. Hcrt neurodegeneration underlies human narcolepsy and is recapitulated in the orexin/ataxin-3 transgenic (TG) mouse model. Acute antagonism of Hcrt receptors has recently been investigated as a novel mechanism of sleep promotion, however, the use of Hcrt antagonists in narcoleptics has not yet been evaluated. Here, we determined the hypnotic and cataplexy-inducing efficacy of Hcrt receptor antagonism by almorexant in the orexin/ataxin-3 mouse model of chronic Hcrt deficiency for comparison with wild type (WT) controls. We also examined the effects of disrupted Hcrt signalling on body temperature (Tb) during sleep. During the 12-h dark period immediately following dosing, almorexant exacerbated cataplexy, decreased wakefulness, and increased nonREM sleep with heightened sleep/wake fragmentation. The antagonist showed greater hypnotic potency in WT than in TG mice. The 100 mg/kg dose of ALM conferred maximal promotion of cataplexy in TG mice and maximal promotion of REM sleep in WT mice. In TG mice, the 30 mg/kg dose of ALM paradoxically induced a transient increase in alertness with elevated motor activity. Tb decreased after acute Hcrt receptor blockade, but the reduction in Tb that normally accompanies the wake-to-sleep transition was blunted in TG mice. These complex dose- and genotype-dependent

interactions underscore the importance of effector mechanisms downstream from Hcrt receptors that regulate arousal state.

Disclosures: **S.W. Black:** None. **S.R. Morairty:** None. **S.P. Fisher:** None. **T. Chen:** None. **D.R. Warrier:** None. **T.S. Kilduff:** None.

Keyword(s): HYPOCRETIN
OREXIN
SLEEP

Support: NIH Grant R01NS057464

Award Number W81XWH-09-2-0081 from the U.S. Army Medical Research Acquisition Activity to T.S.K.

[Authors]. [Abstract Title]. Program No. XXX.XX. 2012 Neuroscience Meeting Planner. New Orleans, LA: Society for Neuroscience, 2012. Online.

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Presentation Abstract

Program#/Poster#: 799.23/BBB24

Presentation Title: [Effects of a dual hypocretin receptor antagonist on sleep and wakefulness in rats.](#)

Location: Hall F-J

Presentation time: Wednesday, Oct 17, 2012, 10:00 AM -11:00 AM

Authors: ***M. D. SCHWARTZ**, L. DITTRICH, S. P. FISHER, W. LINCOLN, H. LIU, M. A. MILLER, D. R. WARRIER, A. J. WILK, S. R. MORAIRTY, T. S. KILDUFF;
SRI Intl., Menlo Park, CA

Abstract: Benzodiazepine receptor agonists promote sleep by activating GABA_A receptors, leading to generalized reduction in cortical activity. They are widely used as hypnotic medications, but have side effects including risk for tolerance and/or dependence, as well as cognitive impairment while under their influence. The excitatory hypocretin (HCRT) neuropeptides promote wakefulness by activating multiple subcortical wake-promoting neurotransmitter systems which, in turn, project to and regulate cortical activity. Blocking HCRT signaling should therefore promote sleep by acting specifically on subcortical brain areas regulating sleep and wake without adversely impacting cortical function. Here, we assessed the ability of the dual HCRT receptor antagonist almorexant (ALM) to promote sleep in rats following ablation of a major sleep-wake regulatory region, the cholinergic basal forebrain (BF). We predicted that ALM would be less effective at inducing sleep in BF-lesioned rats compared to neurologically-intact rats, whereas benzodiazepine-based compounds should be equally as effective in lesioned and intact rats. Male rats received bilateral stereotaxic injections of saline or the selective cholinergic neurotoxin 192-IgG-saporin (SAP) directed at the BF and were implanted with telemetry for recording sleep EEG. Following recovery, animals were given increasing doses of ALM, the GABA-A receptor agonist zolpidem (ZOL), or vehicle. Spontaneous sleep/wake regulation and homeostatic recovery from sleep deprivation was

also assessed. At baseline, NREM sleep in the dark (active) phase was reduced in SAP rats compared to intact rats; SAP rats also exhibited decreased NREM recovery sleep following 6 h sleep deprivation in the dark phase. Sleep in the light (rest) phase was unaffected by SAP. Analysis of ALM and ZOL administration in these animals is currently in progress.

Disclosures: **M.D. Schwartz:** None. **L. Dittrich:** None. **S.P. Fisher:** None. **W. Lincoln:** None. **H. Liu:** None. **M.A. Miller:** None. **D.R. Warrier:** None. **A.J. Wilk:** None. **S.R. Morairty:** None. **T.S. Kilduff:** None.

Keyword(s): SLEEP DEPRIVATION
ADENOSINE
CHOLINERGIC

Support: USAMRAA Grant W81XWH-09-2-0081

[Authors]. [Abstract Title]. Program No. XXX.XX. 2012 Neuroscience Meeting Planner. New Orleans, LA: Society for Neuroscience, 2012. Online.

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